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Volume LXXI Number 3

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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DECEMBER, 1936

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

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Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

Vol. LXXI, No. 3 December, 1936

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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE EFFECT OF ALCOHOL ON GASTRULATION IN ARBACIA

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I

Following certain changes in the environment, the endoderm of echinoderm embryos (*Arbacia*, *Paracentrotus*, *Asterias*, *Echinarachnius*) can be made to develop externally as an exogastrula. Other effects include the development of the endoderm at the expense of the ectoderm and the irregular formation of skeleton and body form. First discovered by Herbst as a lithium effect, exogastrulation has since been shown by several investigators to be caused by a large variety of agents, both chemical and physical (cf. Waterman, 1934). The result is given by fertilized eggs, cleavage stages or blastula stages. Recently exogastrulation has been provoked in another group of animals, the Amphibia. Holtfreter (1933) has found that exposure of axolotl eggs to NaCl solutions caused exogastrulation in some of the embryos, while Curtis et al (1936) have provoked exogastrulation by exposing blastulae of *Amblystoma*, *Rana* and *Bufo* to X-ray.

As alcohols are said to act as depressive narcotic agents, several have been tried to see if gastrulation could be permanently suppressed by them without interfering markedly with subsequent growth and differentiation. While this was not found to be the case, except in instances of fatal injury to the more susceptible individuals, certain effects were observed which are described below. Blastula and young gastrula stages were exposed for about eighteen hours to various concentrations of methyl, ethyl, propyl, isopropyl, butyl, amyl and capryl alcohols. Since individuals of a culture vary widely in their susceptibility to the alcohols, the results are based on the condition of approximately fifty per cent or more of the embryos as determined by the study of at least three random samples from each culture.

In general alcohols act as depressive narcotic agents although they may exhibit exactly opposite effects in different concentrations (Lillie,

1912*a*). In very low concentrations they may stimulate development (Lillie, 1912*a*; Mast and Ibara, 1922, on tadpoles; Elhardt, 1930, on chick) while the higher concentrations are toxic. The higher alcohols are more toxic than the lower ones. Alcohol produces cyclopean eyes in *Fundulus* and also tends to suppress the development and differentiation of the auditory vesicles (Stockard, 1910). Certain concentrations will cause complete but reversible arrest of cleavage (Lillie, 1914). Eggs show a rhythmical susceptibility. The action of alcohol, and other anæsthetics, has been variously interpreted (Lillie, 1916; Henderson, 1930; Winterstein, 1926).

II

Reference to Tables I and II will show the approximate concentrations which produced certain effects. The concentrations are ex-

TABLE I

Influence of alcohols upon gastrulation and subsequent development during eighteen hours of exposure. Blastula stage.

Alcohol	Concentration of alcohol expressed in cc. per 100 cc. of sea water						
	Toleration. No effect on development	Retardation and inhibitory effects	Gastrulation only	Approximate limits of complete but reversible inhibition of gastrulation in some or all individuals	Complete inhibition of any gastrulation	All dead	Simple exogastrulæ which did not differentiate
Methyl	0.2–1.5	1.5–3.5	4 –5.5	5.0–5.9	5.5	6.0	2.5
Ethyl	0.5–0.9	1.0–2.0	2.0–3.6	2.7–4.4	3.8	5.0	0.9–1.3
Propyl	0.1–0.5	0.5–1.0	0.6–1.3	1.4–2.0	1.8	2.0	0.2–0.8
Isopropyl	0.1–1.5	1.6–2.0	2.0–2.4	2.1–3.1	3.2	3.5	1.0–1.7
Butyl	0.1–0.2	0.3	0.7	0.7–1.3	1.0	1.6	0.3–0.4
Amyl	0.1	0.1–0.2	0.3–0.4	0.4–0.6	0.5	0.7	0.1–0.3
Capryl		0.01	0.01–0.02	0.02–0.05	0.04	0.06	0.01–0.02

pressed as cubic centimeters of the alcohol which were added to 100 cc. of sea water containing the embryos. It will be noted that very little if any significant difference exists between the results for the two stages, indicating that they are probably equally sensitive. To avoid repetition only the results secured from the exposure to methyl alcohol will be described. The data for the others are included in the tables.

Methyl alcohol.—The embryos tolerate long exposure to concentrations from 0.2 cc.–1.5 cc. with no appreciable effect on gastrulation and development. In a 2 cc. concentration, development is slightly retarded but plutei develop. In a 2.5 cc. concentration,

gastrulation and subsequent development are further retarded, and after 18 hours fewer top-swimming individuals are present. These are small plutei with frequent abnormal skeletal structures and inhibited arm development. A few undifferentiated cases show slight external outpushing of the vegetal pole cells or rupture in this region. Movement is slower.

In a concentration of 3 cc. gastrulation is further retarded or even inhibited and fewer stunted young plutei (especially with reference to arm development) are lethargically swimming through the medium. Most of the culture is on the bottom of the container. Individuals in which the vegetal pole cells are unevenly extruded or in which rupture, followed by some disintegration of the vegetal pole region, has

TABLE II

Influence of alcohols upon gastrulation during eighteen hours of exposure

Alcohol	Concentration of alcohol expressed in cc. per 100 cc. of sea water			
	Approximate limits of complete but reversible inhibition of further gastrulation in some or all individuals	All dead	Simple exogastrulæ which did not differentiate	Complete inhibition of any gastrulation
	Young Gastrula			
Methyl	3.9–5.5	5.9	0.4–2.5	5.2
Ethyl	2.0–4.0	4.7	0.5–1.5	3.5
Propyl	1.6–1.9	2.1	0.5–1.5	1.8
Isopropyl	2.2–2.6	3.0	0.3–1.4	2.3
Butyl	0.7–0.9	1.3	0.1–0.7	1.0
Amyl	0.4–0.6	0.7	0.1–0.2	0.5
Capryl	0.02–0.03	0.05	none	0.04

occurred are quite common. The ectoderm of the embryos is ragged but the apical plate is present, even in embryos which do not go beyond the late gastrula stage. In the same length of time the control formed medium-sized plutei.

In a 3.5 cc. concentration the embryos develop as far as the triangular stage with differentiation of the apical plate, but lack skeleton. In a 4 cc. concentration the embryos only go as far as the late gastrula in 18 hours. A 4.5 cc. concentration gives shallow or early gastrulæ and their movement is very slow and tends to be rotational, while 5 cc. gives only shallow gastrulæ at the most, and many blastulæ.

Gastrulation is inhibited for the most part at a concentration of 5.5 cc. although a few show initial gastrulation. There is very little movement. On return to fresh sea water after 18 hours in the solution,

these blastulæ gastrulate and continue development. In concentrations from 5.6 cc.–5.9 cc. fewer and fewer blastulæ survive and these gastrulate in fresh sea water, although frequently in an abnormal manner. Subsequent differentiation is abnormal and many do not go beyond a late gastrula stage. In a concentration of 6 cc. the embryos do not survive the experimental period.

Above a minimal concentration of the alcohols the general effect is a depression of gastrulation and subsequent development which is proportional to the kind of alcohol, the concentration and the length of exposure. Methyl is least depressive and capryl the most, while isopropyl is less depressive than the normal alcohol. Lillie (1914) found the latter to be the case in his study of the action of alcohols in suppressing cleavage in sea-urchin eggs. The different alcohols give quite similar results but within progressively narrower limits for the higher ones. The present series was found by Pantin (1930) to hold for the action of alcohols on the ameboid movement of *Amœba* and by Lillie (1914) in the suppression of cleavage.

Gastrulation may be inhibited by alcohol for at least as long as 18 hours without loss on the part of some embryos of the ability to gastrulate and without appreciably detrimental effect on whatever processes are involved. When returned to fresh sea water, the surviving blastulæ gastrulate with apparently undiminished vigor and subsequent development may be quite typical. When developmental abnormalities appear, they are associated with differentiation, i.e., formation of the skeleton, size of the body, and position and length of the arms. These plutei are less resistant and die sooner than do the control. This temporary inhibition of gastrulation is shown by the following observations of embryos exposed to ethyl alcohol which is only slightly more toxic than methyl.

After 5 hours in a 3 cc. concentration the embryos were still in the oval blastula stage. Lethargic movement was visible but all were on the bottom of the container. At this time the controls were at the late gastrula or triangular gastrula. When changed to fresh sea water these developed after 17 hours to the late triangular and early pluteus stages. A few, consisting of the weaker individuals, were at the blastula stage or showed irregular gastrulation with some slight external development of the vegetal pole cells or rupture of the vegetal pole region. After the same length of time in a 5 cc. concentration, no movement was visible and there was considerable evidence of cellular swelling and disintegration especially at the vegetal pole region. Seventeen hours after changing to fresh sea water the embryos were at the late triangular gastrula stage with large skeletal

spicules. At this time the control was at the young pluteus stage. Twenty-four hours later the embryos had developed into normally appearing individuals with no skeletal peculiarities as far as were seen. A 5 cc. concentration was fatal during an exposure of 18 hours.

If inhibition occurs during gastrulation, it is resumed at the point where it left off and goes on to completion with the removal of the alcohol. During the interim nothing vital to the process has been removed, passed over or destroyed. Under the influence of the alcohol the stage of gastrulation does not express itself as determining the capacity to continue gastrulation in the modified environment. Concentrations which inhibit gastrulation at a late cleavage or blastula stage likewise stop it after it has begun or during the process. Thus no difference in sensitivity was demonstrated. Only death or fatal injury completely inhibited typical gastrulation as tested by the ability of the embryos to undergo further development.

Gastrulation is not apparently more susceptible to an anæsthetic like alcohol than other developmental processes. Differentiation without gastrulation was not seen, nor gastrulation without differentiation except in fatally injured embryos. Such embryos live for only a short time after transfer to fresh sea water and gastrulation is not typical. These may show an irregular proliferation of cells at the vegetal pole or the blastocœl becomes packed with cells. The few cases of apparent initial exogastrulation which appeared in lower concentrations and after shorter exposure periods were of this type or showed a very shallow or irregular outpushing of the vegetal pole cells. Only some of the more susceptible individuals in the lower concentrations showed this condition. They did not differentiate beyond forming an apical plate and a small, triangular body form. Frequently the blastocœl was more or less packed with cells.

Abnormal embryos, no matter how produced, always tend to swim in a small circular path. The more retarded or abnormal the structure, the shorter is the diameter of the circle. Modification of shape would account for this in part, but, where embryos are very retarded though otherwise quite symmetrical, there does occur a modification of ciliary distribution and size. The cilia of the blastula and gastrula stages are least sensitive to the narcotic action of alcohol. In concentrations which inhibited development beyond the stage at which it was put into the solution, the cilia continued to beat but at a slower rate. In concentrations immediately above this value, movement was much reduced but it was stopped completely only with complete death or disintegration of the embryo. In lower concentrations, the rate is unaffected but becomes gradually slower above a minimal concentra-

tion. The change does not parallel developmental inhibition. This difference in the narcotizing concentration for different tissues is well known. Lillie (1912*a*) found that the cilia of *Arenicola* larvæ continued their activity for hours in isotonic sugar or magnesium chloride solutions, ether and chloroform, which completely inhibited any muscular movement. In the present study the coördination of the ciliary action was not apparently disturbed although the pathways of the embryos tended towards small circles. Dying embryos did show a fluttering effect attributable to disintegration.

One of the first of the differentiating tissues of the embryo to be affected by the treatment with alcohol is the mesenchyme and this becomes apparent in the modification of the skeleton. This effect has been observed by Runnström (1928) and others (Waterman, 1932, 1934) following the use of LiCl, various other salts, and also physical agents. The modifications first appear in concentrations just above the limit of toleration and are evident in the irregularity and loss of symmetry of the skeletal parts. The ends of the body rods are smooth and unfused. In higher concentrations there appear suppression and reduction of the skeletal rods on one side or both, difference in thickness and amount, accessory parts or projections, and abnormal positions. Alcohol thus markedly affects the distribution and activity of the skeleton-forming mesenchyme, even when the remainder of the embryo may be quite typical in general appearance for the particular stage.

Another modification is that of the fusion of embryos. This varied from pairs up to large aggregations. Only those fusions involving two embryos underwent any considerable differentiation although the members of a large aggregation might gastrulate. The former are comparable to those secured by other means and by other investigators (cf. Waterman, 1932, 1934). Fusion seemed to occur at regions of initial disintegration, and, since this occurred generally in the region of the vegetal pole, many cases failed to gastrulate or did so in a very irregular manner. This region is more susceptible and is the one which seems to be most active physiologically and developmentally at this time (Child, 1928). The swelling and partial separation of the blastomeres in this region point to an injurious permeability change. The final result is an irregular mass of swollen, spherical cells which gradually disintegrates.

That weak solutions of the alcohols may give a slight acceleration of development seems to have been found in the course of this study. This was seen to occur with methyl, ethyl and isopropyl alcohols in the lowest concentrations employed. In the case of the earliest stage

the embryos for the most part gastrulated sooner, and in all cases the plutei matured more quickly than the control. They did not survive as long as the control. There was, however, no noticeable difference in final size. Others have likewise found that weak solutions of certain alcohols accelerate development. In a study of the effect of ethyl alcohol on tadpoles, Mast and Ibara (1922) found that, although there was a reduction in activity of the tadpoles, there occurred an increase in the rate of growth and a decrease in the rate of mortality. While it is not clear as to how these effects were produced, they suggest that the reduction in activity might account for the longer life and that the alcohol served as food which conserved the reserve food material in the tadpole's body. The animals were not fed during the experiment. Alcohol may also increase irritability when used in low concentrations, while exhibiting typical anæsthetic action in higher concentrations. Reference should be made to Lillie (1912*a*), page 374, for a discussion of this point and additional examples. More recently Elhardt (1930) has found that young chicks respond by an increased growth rate to doses of ethyl alcohol. Methyl, propyl and butyl alcohols (1932) had a stimulating effect on growth for a time.

Summary

The general effect of immersing the blastula and young gastrula stages in various concentrations of methyl, ethyl, isopropyl, propyl, butyl, amyl and capryl alcohols for 5–18 hours is a depression of gastrulation and development which is conditioned by the kind of alcohol, the concentration and the length of exposure. They all give quite similar effects but within narrower limits for the higher ones.

Apparently the process of gastrulation is no more sensitive to the alcohols than other developmental processes. It may be inhibited for 5–18 hours without the ability or vigor of gastrulation being diminished. The stage of gastrulation does not express itself as determining the capacity to continue gastrulation in this modified environment. Differentiation without gastrulation was not found nor gastrulation without differentiation except in fatally injured embryos. Information is given on the concentrations which caused complete but reversible inhibition of gastrulation and on the limits of alcohol toleration. A slight acceleration of the rate of development was observed in the lowest concentrations of methyl, ethyl and isopropyl alcohols.

Embryonic differentiation is affected by the alcohols. Abnormalities appeared in the development of body form and size, length of the arms, and structure and amount of the skeleton. Ciliary activity is

not inhibited in concentrations which stop development but the rate is reduced. Fusions appeared in the cultures but only those involving two individuals underwent any considerable differentiation. Few exogastrulæ were found and they were always of a shallow type which did not differentiate to any extent. This abnormality appeared among the more susceptible individuals in the lower concentrations of the alcohols.

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REGENERATION IN MNEMIOPSIS LEIDYI, AGASSIZ[1]

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The only report giving results of experiments dealing with regeneration in ctenophores has been made by Mortensen (1913). His experiments, which were done on only four specimens of *Bolina infundibulum*, served to demonstrate that these animals possess the power to regenerate lost parts. These experiments of Mortensen, as well as my observations of regenerating specimens made incidentally while collecting *Mnemiopsis*, disprove the common conception that ctenophores will not regenerate. These observations led to experiments which deal with the regeneration of organs and other parts that have been removed from the body of a ctenophore, *Mnemiopsis*. It is the purpose of this report to present these experiments. Further investigation which may give solution in part to problems depending on some aspect of regeneration in this animal will be reported at a later date.

Methods

The specimens were measured along the three main axes of the body and cut into parts with scissors. Each piece was kept in a separate finger bowl during the period of regeneration. The finger bowls were partly immersed in running water in order to maintain a fairly low and constant temperature. The temperature remained near 21° C. during most of the period of observation, reaching a low of 18.9° C. and a high of 23° C. This range of temperature was satisfactory since the survival of the regenerating pieces was high. These pieces were observed through a dissecting microscope at frequent intervals to note the progress of regeneration.

All of the photographs shown in this report were taken with a Leica camera set up with an extension tube outfit. A black background was placed beneath the specimens and out of the focal range of the lens. The lighting consisted of one Spencer microscope lamp placed in a horizontal position and one photoflood lamp placed above the specimens. These animals were photographed while in a large

[1] Contribution No. 16 from the Department of Biology, Brooklyn College.

finger bowl filled with a solution of sea water and a low percentage of alcohol. The alcohol stopped the movements of the animal except those of the paddle plates. The rate of movement of these plates was decreased sufficiently to allow time exposures.

Experiments

The sequence of recognizable phases during regeneration in *Mnemiopsis* was wound closure, swelling and stretching, and reformation of lost parts. Wound closure was accomplished by the edges of the outer body wall moving together and fusing at the cut area. At this time the corresponding canals and rows of plates fused with each other. Wound closure was complete five hours after the operation. The swelling and stretching of the region at the regenerating area was indicated by the stretching of the rows of plates (Fig. 1). Stretching was complete within 18 hours and at this time the plates on these rows had become widely separated (Fig. 1). Plates regenerated on the rows at regular intervals between the old plates within a few hours. When cuts were made to remove parts of the body containing rows of plates or the apical organ, these structures were reformed. In the sequence of regeneration of organs which had been removed, the apical organ formed first and the regeneration of rows of plates followed within a few hours. Plates regenerated on these rows within three hours after the rows were complete. The experiments reported here are grouped according to the following headings: (1) regenerating halves; (2) regenerating thirds; (3) regenerating fourths and eighths.

Regenerating Halves

Specimens were divided into halves by a cut across the body midway between the apical and oral ends, by a longitudinal cut midway between the adesophageal plate rows (*a–b*, Fig. *A*), and by a longitudinal cut midway between the adtentacular plate rows (*c–d*, Fig. *A*).

Regeneration following the cutting across between the apical and oral ends gave the following results. New plates were formed between the old ones along the stretched rows at the oral end of the apical pieces within five days. Additional plates were formed also at the apical end of the oral pieces within three days. An apical organ was formed in most of the oral pieces within two days.

The specimens which were divided into halves along the long axis of the body between the adesophageal plate rows were cut at the midline except in the region of the apical organ. In this region the cut was made just to one side of the apical organ in order to leave this organ in one of the halves. Fifteen of the 20 halves which retained the apical organ lived and each regenerated 4 complete rows of plates

within 9 days. Two views of one of these regenerating halves are shown in Figs. 2 and 3. Of the 13 halves which lived and which did not retain the original apical organ, 3 reformed this organ and 4 complete rows of plates within 11 days, 4 rounded up and formed the apical organ without regenerating any rows of plates, and 6 showed no

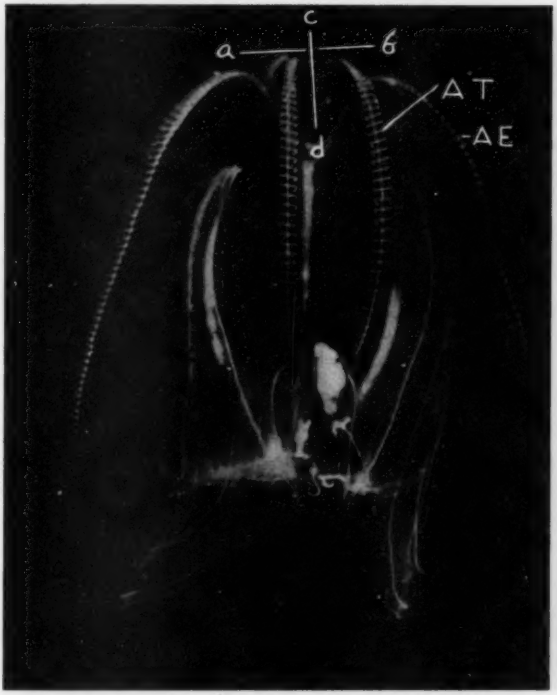

FIG. *A*. This is a photograph of a living animal. It shows some of the internal organs and above it are lines to show planes through which cuts were made. This specimen was fed some clam juice and this food caused the internal organs to show. Some of this food can be seen near the mouth of this specimen. Lines *a–b* and *c–d* show the adesophageal and the adtentacular planes respectively. *AE*, adesophageal row, and *AT*, adtentacular row.

signs of regenerating either an apical organ or rows of plates by the end of the tenth day. The halves which formed the apical organ without regenerating any rows of plates rounded up and behaved as normal specimens.

The animals which were divided into halves along the long axis of the body between the adtentacular plate rows were cut at the mid-

line except in the apical region. In this region the cut was made to one side of the apical organ in order to leave this structure in one of the halves. Eleven of the 20 pieces which retained the apical organ lived and each regenerated 4 complete rows of plates within 8 days. Only 5 of the 20 pieces which did not retain the original apical organ lived and of these one formed this organ and 4 complete rows of plates while the remaining 4 specimens failed to regenerate either an apical organ or rows of plates.

Regenerating Thirds

In this experiment the animals were divided into thirds by two cross cuts between the apical and oral ends of the body. By these cuts each specimen was divided into an apical piece, a middle piece, and an oral piece. Each of the 15 apical pieces survived and formed the row and canal connections as well as lobes and auricles at the oral end. Twelve of 15 mid-pieces lived and regenerated the apical organ with the regular canal connections in the apical zone (Fig. 1), and canal connections as well as lobes and auricles in the oral end. Each of the 13 oral surviving pieces regenerated an apical organ and formed the regular canal connections. Regeneration was complete in all apical, middle, and oral pieces within 7 days.

Regenerating Fourths and Eighths

Specimens were divided into fourths by three methods of cutting. According to one method two cuts were made at right angles to each other, one between the adesophageal plate rows and the other between

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EXPLANATION OF PLATE I

All photographs in this plate were made of living animals. *A*, apical organ; *AU*, auricle; *M*, mouth region; *P*, plate; *PR*, plate row; and *S*, stomodeum.

FIG. 1. This shows the apical end of a regenerating mid-piece of an animal which had been cut into thirds. The stretched rows are shown at *PR* with the separated plates at *P*. Later additional plates were formed on these rows between the old plates.

FIG. 2. This shows a regenerating half of a specimen which had been cut along the long axis of the body between the adesophageal rows. The regenerated rows of plates can be seen at *PR*.

FIG. 3. This is a view of the normal surface of the animal half shown in Fig. 2. The old rows can be seen clearly.

FIG. 4. This is a photograph of an apical quarter of a specimen showing the regenerating surface. This piece was produced by cutting a specimen across between the apical and oral ends and by another cut between the adtentacular rows. The piece shown here retained the original apical organ. Note the regenerating rows of plates.

FIG. 5. This is a photograph of an oral quarter of a specimen which had been cut as the one shown in Fig. 4. This piece did not regenerate any rows. It rounded up after regenerating an apical organ and behaved as a normal animal.

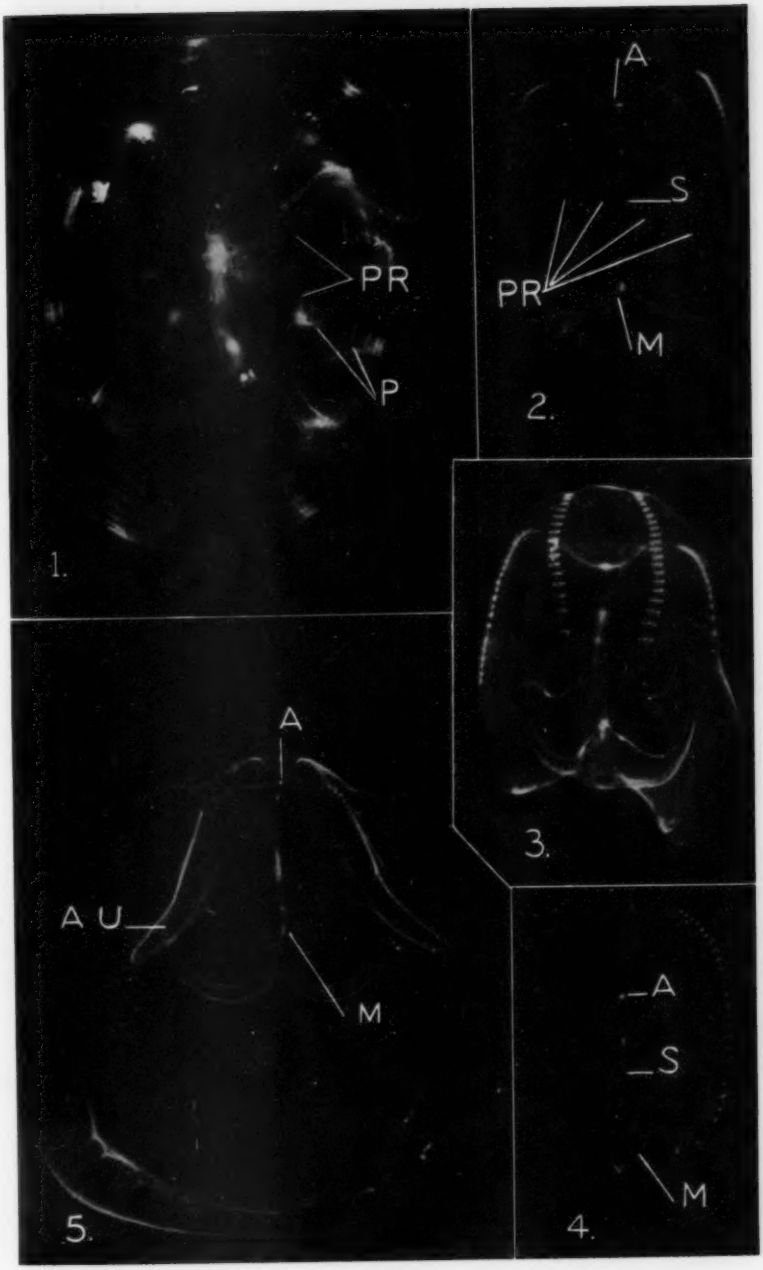

PLATE I

the adtentacular plate rows, through the longitudinal axis of the body. By another method one cut was made across the animal midway between the apical and oral ends and each of these halves was cut longitudinally between the adesophageal plate rows. The third method was similar to the second one except that the longitudinal cut was made between the adtentacular plate rows.

The following is the result of regeneration in the pieces produced by the first method of cutting. Sixty-five of 80 pieces survived and 5 of these formed 6 rows of plates and an apical organ each within 7 days. Of the 32 pieces which failed to regenerate by the eleventh day only 3 had retained the original apical organ. Each of the remaining 28 pieces formed from one to four plate rows within 7 days. The experiment was discontinued on the eleventh day and at this time each of the 28 pieces showed definite signs of regenerating completely the parts which had been removed by the operation.

Four of the 14 fourths retaining the original apical organ which were produced by the second method of cutting formed 4 rows of plates within 7 days. Seven of the 14 pieces healed and failed to regenerate any rows of plates but rounded up and behaved as normal animals. The remaining 3 pieces failed to round up or to regenerate any rows of plates. Four of the 12 apical pieces which did not retain the original apical organ formed this organ with canal connections within 7 days. Plate rows were not formed during this time and each of the specimens reacted as normal animals. One of the apical pieces regenerated both an apical organ and 4 rows of plates within 7 days. Each of the remaining 12 pieces failed to regenerate either plate rows or an apical organ and when they were discarded at the end of the seventh day they showed no evidence of regeneration. Of the 22 oral fourths, 11 regenerated the apical organ and rounded up to form individuals which behaved as normal animals though with only the 4 original rows of plates. All of the remaining oral pieces failed to regenerate either the apical organ or the rows of plates.

All of the 15 apical quarters produced by the third method of cutting and which retained the apical organ survived and 6 of them regenerated 4 complete rows of plates (Fig. 4) while each of the remaining 9 pieces rounded up without regenerating any of the lost rows of plates and behaved as a normal animal. Only 9 of the 15 apical pieces which did not retain the apical organ lived and 8 of these formed this organ, rounded up and continued to live without further regeneration. The remaining one of the apical pieces regenerated both the apical organ and 4 rows of plates. Twenty-seven of the 30 oral pieces survived and of these only one regenerated both the apical

organ and 4 rows of plates, 18 regenerated only the apical organ and rounded up (Fig. 5), while 8 failed to regenerate either the apical organ or any rows of plates.

Eighteen specimens were cut into fourths by cuts passing through the adesophageal and the adtentacular planes. Each fourth was then cut across midway between its apical and oral ends to produce eighths. All of these eighths died within 28 hours and at this time had given no evidence of regenerating.

Discussion

The results of experiments whereby various parts of the body of *Mnemiopsis* were reformed show conclusively that these animals will regenerate the lost parts. This in itself is significant since ctenophores have been regarded as a group without the power of regeneration. The rapidity of regeneration in *Mnemiopsis* and the transparency of its body which makes observations clear and convenient are also important features possessed by this animal. Experiments on *Mnemiopsis* show further that this animal possesses a regulating center. The halves and fourths produced by longitudinal cuts which retained the apical organ regenerated the rows of plates within less time and in a higher percentage of cases than those pieces which did not retain this organ. Furthermore, in all portions of *Mnemiopsis* which did not retain the original apical organ this structure preceded the rows of plates in regeneration in all cases. I therefore conclude that the apical organ in *Mnemiopsis* is the regulating center during regeneration. The apical organ previously has been considered (Coonfield, 1936) as a region of dominance and the conclusion expressed in this report gives further proof that this organ constitutes an important center in this animal.

It is interesting to note that following longitudinal cutting in *Mnemiopsis* certain pieces failed to form the lost rows of plates, rounded up and continued to live as normal animals. Some of these pieces had retained the original apical organ while others were without this organ after the cutting. Those pieces which did not retain the original apical organ soon regenerated this structure. It is clear then that all of these pieces which rounded up were unable to carry out reconstitution to completion.

Conclusions

1. Halves, thirds, and fourths of *Mnemiopsis* regenerated the lost parts while eighths of this animal failed to regenerate.

2. Some of the halves and fourths failed to regenerate rows of plates and these pieces continued to live as normal animals.

3. The sequence of regeneration of organs was the apical organ, rows of plates, and plates on these rows. The plates were complete within three days after the formation of the rows.

4. Certain evidences brought out by regenerating pieces of *Mnemiopsis* show that the apical organ of this animal is the regulating center.

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SENSORY HAIRS OF THE DOGFISH EAR

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Sensory hairs from the crista of the fresh water teleost, *Ameiurus nebulosus*, have been viewed in living condition and observations have been recorded on the nature and distribution of these hairs (Bowen, 1932). The conclusions reached through the study of such living materials are different in some significant features from those which have been arrived at, by different investigators, from examination of preserved ampullæ.

The internal ears of elasmobranchs have been the subject of numerous investigations and the general structure of those in the dogfish is well known (Retzius, 1881). Due to their comparatively large size and ready accessibility, the internal ears of dogfish have been studied repeatedly in relation to equilibrium (Maxwell, 1923). Examination of the sensory endings in the labyrinth, and particularly the sensory hairs of the crista in elasmobranchs, however, has been limited to preserved materials.

In order to determine more accurately the nature of the ampullar organs in the internal ear of elasmobranchs, observations have been made of fresh ampullæ from the dogfish, dissections for these studies being made according to the technique which has been described for *Ameiurus*. This paper deals with the sensory hairs of the cristæ as they appear in living condition in their natural medium, endolymph, and in media differing somewhat in composition from such endolymph.

These observations were carried on for the most part in the Marine Biological Laboratory, Woods Hole, Massachusetts. Records of the examinations were secured with photomicrographic apparatus made available through the generosity of the Elizabeth Thompson Science Fund.

In order to determine with some precision the concentration of fluid necessary for maintaining the hair cells of the dogfish ear as in the intact animal, it was found necessary to examine cristæ in different media and to note the effects of each upon the sensory hairs. Cristæ, therefore, were examined in the following solutions: Ringer's solution of varying concentrations; different dilutions of sea water; distilled

water; sea water itself; sea water to which additional sodium chloride had been added.

Initial dissections of the crista, for which *Mustelus canis* and *Squalus acanthias* were employed, were made in Ringer's solution and in dilutions of sea water approximating Ringer's solution in concentration (United States Bureau of Fisheries Laboratory and Harvard Zoölogical Laboratory). Similar dissections of cristæ from the ampullæ of four sharks of the species *Carcharius commersonii* were made at the Bermuda Biological Station. These preparations, dissected in media of saline content less than sea water, consistently failed to show sensory hairs in any condition approximating those found in *Ameiurus*. In most of these cristæ no sensory hairs whatever could be found. In others there were bare traces of the projecting hairs, these being reduced to a small fraction of the length of sensory hairs such as are found in *Ameiurus*.

Through repeated trials with numerous later dissections it has been found that the sensory hairs of *Mustelus canis* maintain their size and appearance to best advantage when they are kept bathed in sea water and not in other solutions of higher or lower concentrations. Deviation from the concentration furnished by sea water tends to produce decided alterations in the sensory hairs. In solutions slightly lower or slightly higher in saline content there is a reduction in the number of hairs and with greater deviation a further reduction in size and number of these structures. Fresh dissections in sea water, however, invariably show the sensory hairs in good condition.

The sensory hairs, when viewed in cristæ removed from the ampullæ in sea water and held in such medium for observation under the higher magnifications, are found to be slender, tapering processes which extend at right angles from the sensory surface. The sensory hairs of the dogfish range between 35 μ and 60 μ in length, the latter figure representing the length of the greater number of the undisturbed processes. In *Ameiurus* the length of the sensory hairs is near 50 μ. The slight deviation of the hairs, near the middle of the crista, from the midpoint toward either end, a structural feature which was recorded for *Ameiurus*, is not noted in the dogfish.

The sensory hairs of the dogfish crista are, in some instances, maintained without appreciable alteration for a matter of hours in sea water. After three or four hours degenerative changes may appear and, in some preparations, the hairs have been seen to shorten and become reduced in number. Complete disappearance occurs some hours later.

In preparations which have been kept for approximately an hour in sea water or for a shorter time in solutions of slightly greater or less saline content than sea water, protrusions of clear, mucus-like material form along the surface of the sensory layer. In a short time this substance increases in amount and globules of the material, becoming entirely detached from the neuroepithelial surface, remain near the bases of the sensory hairs. This material seems to be rather of the nature of a secretion than a product of cytolysis, for if, in the early stages of its formation and before the destruction of the hairs occurs, the crista is washed in sea water, the globules are removed though the sensory hairs remain. It has not yet been found possible to determine definitely whether these materials arise from the sensory cells or from the supporting cells. The position of the globules, when they first appear near the bases of the sensory hairs, suggests that the materials are coming from the supporting cells.

The hairs in one preparation were found to be moving. In this case the crista was first observed under high magnification about half an hour after the decapitation of the fish. Sensory hairs were moving like cilia when the crista was first seen and continued in motion for about thirty minutes from the time of the first observation. This fact in itself indicates that the hair, in sea water, is in a medium which does not immediately cause marked injury to it.

It has recently been found possible in two cases to secure views of the sensory hairs of the crista without removing the ampulla from its position in the chondrocranium and with comparatively little injury to the fish. In these dissections a small opening was cut in the cartilaginous capsule immediately above the left anterior ampulla and the perilymph drawn off with a fine pipette. A slit was then made in the roof of the ampulla immediately above the crista and the walls of the ampulla held away from the field of vision by means of fine glass needles. The sensory hairs could be seen, somewhat dimly it is true, but nevertheless recognizable, through the use of vertical illumination. The cristæ in these two cases were in living animals, in endolymph relatively undisturbed and with the eighth nerve intact. Though optical conditions were not suitable for detailed examination, enough could here be determined as to size, shape and arrangement of the sensory hairs to indicate that the preparations in sea water closely approximated the normal.

The cristæ of the dogfish ear, though similar to those of *Ameiurus*, show some differences, the significance of which must await further investigation. In *Ameiurus* the crista extends slightly farther into the ampulla and the sensory layer forms more nearly a straight line

across the lumen. Whether this more complete blocking of the lumen in *Ameiurus* is of functional significance is not known. The position of the hairs extending directly outward from the sensory layer both in the dogfish and in *Ameiurus* is contrary to the usual description and is a factor which must be considered in any final explanation of the rôle of the crista in equilibrium.

Likewise, the movement of the sensory hairs of the cristæ (Bowen, 1931, 1932, 1935) may have some definite function in the physiology of the labyrinth. What that function may be is still far from evident. The movement of cilia within the cavities of the internal ear of *Petromyzon*, first mentioned by Ecker (1844) has been nicely demonstrated by de Burlet and Versteegh (1930) and the structure of the ear given in much detail. The cilia, found by these investigators to be moving in *Petromyzon*, appear distinct from the sensory hairs of the cristæ. Whether there is any structural or functional relationship between the ciliated epithelium of the cavities and the sensory hairs of the cristæ in *Petromyzon*, and whether the latter are ever in motion are questions not yet answered.

Finally, the formation and appearance of the minute globules, which have been seen to arise among the bases of the sensory hairs in the dogfish ear suggest that material so formed may be a secretion. These globules apparently do not represent disintegration products since they can be washed away, after which the tissues which have been covered with them remain normal. Though the present evidence is indicative only, it is quite possible that this material, arising as a secretion from the neuroepithelium, goes to make up a part, if not all of the cupular substance, a substance which under the influence of fixing reagents forms the cupula of common description.

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THE MORPHOLOGY AND BEHAVIOR OF THE INTRACELLULAR BACTEROIDS OF ROACHES[1]

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The intracellular bacteroids of roaches, some ants, beetles, and other invertebrates, as well as the intracellular yeast-like bodies of aphids, coccids and other arthropods, have been the subject of much work and speculation, ably reviewed by Buchner (1921, 1930) and Glaser (1930). Their morphology and parts of their life cycles have been studied by Mansour (1934), Lilienstern (1932), Koch (1931), Meyer (1925), Florence (1924), Buchner (1912, 1923) and others, but in many of these studies critical points have been passed over because of insufficient material or technical difficulties. It has been definitely established, however, that these intracellular bodies occur in all individuals of the host species and are regularly transmitted to the offspring.

Most workers in the field agree that these bodies are bacteria in some cases and yeasts in others, but some biologists yet prefer to interpret them as mitochondria or waste products as did Cuénot (1896) and Henneguy (1904). Cultivation of the bacteroids on artificial media has been attempted (Mercier, 1907; Hertig, 1921; Glaser, 1930; and others) with negative or questionable results in most cases. My own work on cultivation, which to date is negative, will be published later. If these bacteroids can be cultivated outside the invertebrate host, they must be bacteria; if they cannot be cultivated, they may be bacteria of the same order as *Rickettsia,* they may be other very highly specialized bacteria, or they may be non-living inclusions. We can, however, put considerable reliance on studies of their morphology, staining reactions, and behavior.

The physiology of these intracellular bodies is entirely unknown. One group of workers (Buchner, Florence, Schwartz, and others) considers the relationship to be a true symbiosis between intracellular "symbiont" and invertebrate "host." Another group (Glaser, Mansour, and others) thinks of the relationship rather as being one of commensalism or mild parasitism. Neither faction, however, has advanced any substantial evidence for its contentions. In view of this situation

[1] Contribution No. 252 from the Zoölogical Laboratory, Indiana University. Submitted in partial fulfillment of the requirements for the degree, Doctor of Philosophy.

it seems desirable to avoid such terms as "symbiont," "symbiosis," "parasite," and "parasitism" and the implications arising from their usage, and to designate the intracellular bodies in roaches merely as "bacteroids."

Although the roaches were among the first insects found to harbor intracellular bacteroids (Blochmann, 1888) and there have been reports on various phases of their morphology, staining reactions, and life cycle, no detailed unified work on these bacteroids has been published. Heymons (1895) followed their behavior in a general way while studying the embryology of several species of roaches; Mercier (1907), Buchner (1912) and Fraenkel (1921) added materially to our knowledge of their morphology and life cycle in *Periplaneta* (*Blatta*) *orientalis;* Neukomm (1927) studied their morphology and staining reactions in *Blattella germanica;* and others have contributed details now and then. There is, however, little agreement among the workers and many points have been untouched. Concerning the present knowledge of the bacteroids of roaches, Glaser (1930*a*) wrote, "Many details in the chain of events are entirely lacking, and if known would undoubtedly assist much in the proper interpretation. . . . How non-motile elements are transported along definite, and in many cases, at least, rather sluggish, channels and find their way from the adult host cells to the immature ova and return is entirely unknown."

Materials and Methods

The work was concentrated on the large house roach, *Periplaneta americana,* collected at Pittsburg, Kansas, 400 embryos and 100 nymphs and adults being examined. Over 100 embryos and 80 nymphs of *Parcoblatta pennsylvanica,* collected at Pittsburg, Kansas, and Bloomington, Indiana, and smaller numbers of the following species were studied: *Parcoblatta uhlerania, P. latta, Blattella germanica,* and *Blatta orientalis,* collected at Bloomington and Winona Lake, Indiana; *Periplaneta australasiæ* and *Eurycotis floridana* from Dunedin, Florida; and *Cryptocercus punctulatus* from Knoxville, Tennessee. Many of the specimens were identified by Dr. J. A. Rehn, and the remainder by Dr. W. S. Blatchley.

The roaches were kept in the laboratory in closed metal or glass containers. Water was supplied either in a shallow dish or by wet filter paper. *Cryptocercus punctulatus* was fed filter paper and partially rotted pine wood; all others were fed potato, banana, and a little raw beef.

Eggs for embryological studies were obtained and cared for as follows. As soon as a female was observed to be ovipositing, she was

placed in a separate container. If the oötheca was not dropped within 36 hours it was released by a gentle twist and the roach returned to the breeding pen. The oöthecæ were placed in Syracuse watch glasses in a larger container with a high relative humidity. The eggs of *Periplaneta americana* were incubated at 33° C.; others were left at room temperature. Under these conditions, the incubation time was approximately 30 days for *Periplaneta americana,* 36 days for *Parcoblatta pennsylvanica* and 40 days for *Blatta orientalis.* The oöthecæ of *Blattella germanica* were removed from the females at the time they were to be fixed.

Cytological methods were employed for studying the bacteroids in the gonads and fat tissues. Most of this material was fixed in Flemming or Champy, sectioned in paraffin at 5 μ, and stained in Haidenhain's iron hæmatoxylin. Other fixatives and stains were used as checks. Living material, with and without vital stains, was studied in considerable quantities.

Mature ovarian eggs were fixed in Flemming 12 to 18 hours or in Carnoy-Lebrun 10 to 12 hours and sectioned by the method described by Slifer and King (1933). These eggs were stained either with Giemsa (Wolbach, 1919), or better by Delafield's hæmatoxylin 10 to 15 minutes and destained until only the chromatin and possibly the yolk retained some color, followed by anilin safranin 12 to 20 hours and differentiated in 95 per cent alcohol. The latter method stains the chromatin black, bacteroids purplish red, and yolk pink. Other fixatives and stains were tried with varying success, but most fixatives make the yolk too brittle, and most stains have a greater affinity for the yolk than for the bacteroids.

The following method was adopted for fixing and staining embryos of all stages. The oötheca was opened slightly and dropped into Carnoy-Lebrun fixative. After two hours, the halves of the oötheca were separated, the unbroken chorions opened and the embryos returned to the fixative for a total of 20 to 24 hours. After washing with 95 per cent alcohol, each unbroken embryo was carefully removed from the oötheca and as much of the chorion teased off as possible. The embryos were soaked in 85 per cent alcohol containing 4 per cent phenol, dehydrated, embedded in paraffin, trimmed, and soaked in water 36 to 48 hours as recommended by Slifer and King (1933) for grasshopper eggs. The embryos were sectioned at 5 μ and stained with Giemsa, Wolbach's method.

Carnoy-Lebrun solution has the advantages of penetrating rapidly and at the same time removing the oil globules which become very hard if fixed in the yolk. The yolk is left rubbery by long fixation in Carnoy-

Lebrun although it is made very brittle by most fixatives. It usually becomes so hard during the process of dehydration and embedding that it will not section by the collodion-paraffin method or otherwise without long soaking. After Carnoy-Lebrun fixation, the hardening is not so severe and the soaking process is more effective than after most fixatives. Water penetrates the yolk at the rate of approximately one millimeter in 24 hours at 25° C., softening and slightly swelling it. Giemsa stain, well differentiated, gives fair contrast; chromatin blue-black, cytoplasm pale blue, yolk pink, and bacteroids blue-green. The Delafield hæmatoxylin-safranin method is unsatisfactory to demonstrate clearly the bacteroids in the embryo, as are all other stains tried.

Direct smears of fat bodies, gonads and embryos were used extensively for studying the morphology and checking for the presence of the bacteroids. Such smears were usually dried in air, fixed with heat and stained by Gram or Giemsa. Some were fixed in ether-alcohol, Bouin or Flemming while still wet, and stained as above. Other bacteriological stains were tried with less satisfactory results.

Morphology and Staining Reactions

The intracellular bacteroids of *Periplaneta americana* are slightly curved rods, .9 to 1 μ in diameter and 1.5 to 6.5 μ in length (average 3.5 μ), with round or slightly pointed ends. Spherical forms are of rare occurrence, most of the apparent cocci being ends of rods. In smears fixed in ether-alcohol, and occasionally in other fixatives, there is a distinct enlargement of one end of the rod, producing a club effect (Glaser, 1930*a*), but this is probably due to uneven shrinking and swelling in the fixative. No distinct size variation in the bacteroids has been found in different roaches of the same species.

The rods are either solid or have alternate dark and light bands (Fig. 2). The banded structure is most distinct in air-dried smears stained in Giemsa, and sections of fat-body or ovary fixed in Champy or Benda and stained with Haidenhain's iron hæmatoxylin, well extracted. Smears stained by most bacteriological methods show the banded effect indistinctly. Sections from material fixed in Carnoy-Lebrun, Bouin, Zenker, and other acetic acid fixatives show the banding in approximate inverse proportion to the amount of acetic acid in the fixative. In such material, many of the rods appear to be mere shells, while others have light bands (Fig. 2*b*).

The general size and appearance of the bacteroids is the same in all species of roaches studied. In *Blatta orientalis* and all *Parcoblatta* species studied, however, they are more slender, averaging about .9 by 3.5 μ, while in *Blattella germanica* they are shorter, thicker (1.1 by 3 μ)

and more nearly straight. These differences may be specific but such different roaches as *Periplaneta americana* and *Cryptocercus punctulatus* have bacteroids morphologically very similar.

The bacteroids obviously increase by transverse fission, as all stages of the process (Fig. 2) can readily be found in ovaries and embryos, although the division has not been observed directly. The constriction occurs regularly between two dark bands, dividing the original rod into two equal or unequal parts. The cocci and small buds mentioned by Glaser (1930*b*) have not been observed. Two, three or even four members may remain attached to make a chain as much as 15 μ long. The tendency to form chains is especially pronounced in *Parcoblatta*.

The bacteroids are non-motile, not encapsulated and not spore-forming. They are Gram-positive but not acid fast according to Glaser (1930*b*) and Neukomm (1927). With Sterling's Gram stain, however, they lose the violet completely after 80 to 90 seconds in alcohol, and with Kopeloff and Beerman's Gram stain they are usually negative, while *Bacillus subtilus* and *Staphylococcus aureus* mixed with the bacteroids retain the violet. They stain well with crystal violet, basic fuchsin, and mixtures containing either of these, but stain very poorly with methylene blue in any mixture. They stain readily with Giemsa, the periphery and bars becoming almost black in smears or deep blue-green in sections after differentiation in alcohol. They stain deeply with Haidenhain's iron hæmatoxylin, the intensity of the stain varying wth the fixative used. They do not give the Feulgin reaction to any definite degree. With Altman's acid fuchsin-methyl green the bacteroids stain green or blue-green, the chromatin green and the mitochondria red. The periphery of the bacteroids darkens slightly and unevenly after prolonged treatment in osmic acid.

In living material, the bacteroids are readily visible without stain. They do not take neutral red although the mycetocytes become pink after a short time. They stain distinctly with Janus green B in dilutions up to 1 to 100,000, but are not well differentiated with this stain at any dilution. Cowdry and Olitsky (1922) found no bacteria that stain in Janus green B in dilutions greater than 1 to 60,000, but mitochondria stain perfectly in dilutions as great as 1 to 500,000. They also found that mitochondria do not stain with Giemsa after any fixative used, nor with any stain used after alcohol or Bouin fixation.

With respect to morphology and reactions to fixatives and stains, the intracellular bacteroids and bacteria are very similar, to say the least. Neukomm (1927*b*) found that the reaction of the bacteroids of *Blattella germanica* to ultraviolet rays was likewise similar to that of bacteria. Glaser (1930*b*) has carried this analogy further, apparently with good

reason, and has placed the bacteroids of the roaches taxonomically with the diphtheroids (genus *Corynebacterium*).

Life Cycle

The following descriptions of the locations and behavior of the bacteroids throughout the life cycle of the roach agree in general with those given by Heymons (1895) and Buchner (1912). Many details are added, however, and some discrepancies in the works of former authors are clarified.

In roaches of both sexes, the spaces around the abdominal viscera are more or less filled with a lobulated fat body, well supplied with tracheæ. Each fat body lobe is made up ordinarily of two types of cells: a central row (mycetocytes) whose cytoplasm is packed with the bacteroids, and an outer layer of cells filled with fat globules and urate crystals, completely surrounding the mycetocytes (Fig. 3). Some lobes have two layers of cells around the mycetocytes: an inner layer filled with urate crystals which are usually dissolved during the process of fixation leaving the cytoplasm clear, and an outer layer of regular fat cells (Fig. 4; Cuénot, 1896, his Fig. 2). In thicker portions of the fat body there may be several rows of mycetocytes, interspersed with urate cells or fat cells and completely surrounded with fat cells. In *Periplaneta, Eurycotis,* and *Blatta,* many lobes are thin with only one row of mycetocytes. In *Blatella, Parcoblatta* and *Cryptocercus* more lobes are thicker, with two to twenty mycetocytes in a cross-section.

Mycetocytes, unaccompanied by fat cells, are present in the connective tissue covering both ovaries and testes, but no bacteroids have been found within the testes. In the ovaries of all species studied, however, there is a layer of bacteroids around the larger oöcytes, the relative thickness of which, in *Periplaneta americana,* is shown in Fig. 1.

Before attempting to discuss the relations of the bacteroids to the oöcytes, it seems best to describe some salient points of the structure of the ovary.

An ovary of *Periplaneta americana* is made up of eight tapering ovarioles each of which consists of three parts: an anterior "terminal filament" which anchors the ovary to the body wall, an elongated egg tube, and a short tubular pedicel connecting the posterior or basal end of the egg tube to the oviduct. The entire ovariole is enclosed in a tough, non-cellular membrane, the tunica propria, outside of which is a thin covering of connective tissue and mycetocytes.

The egg tube is divided into two parts: an anterior "germarium" or "tip" made up of oöcytes in the early stages of differentiation; and

the " zone of growth " or " vitellarium " in which the oöcytes grow to maturity (Fig. 1). The most anterior cells in the zone of growth, designated in this paper as the " distal oöcytes," are distinctly larger and more rounded than those in the tip. As the distal oöcytes enlarge, the follicle cells scattered between them multiply and gradually form a complete epithelium around each oöcyte.

The oöcyte at the base of each ovariole reaches a maximum length of .7 mm. before the last moult, after which it enlarges rapidly to 1 mm. in length, then more slowly to about 2 mm. if copulation is not effected. Within an average of eight days after copulation, the basal oöcyte reaches its final size (1 by 3 mm.), becomes encased in a chitinous-like chorion and is oviposited. Meanwhile, the next three oöcytes anteriorly have increased to 1 mm., .6 mm. and .5 mm. in length respectively. Oviposition of the basal oöcyte seems to be the stimulus for more rapid enlargement of the next in line, so there is a succession of mature eggs at intervals of approximately eight days.

No bacteroids have been found in the germarium. There are usually a few in the angles between the distal oöcytes, and gradually increasing numbers more posteriorly, pressed closely against the oöcyte membrane (Fig. 5). The follicular epithelium forms outside the bacteroid layer and splits the layer between oöcytes thus enclosing a separate layer around each oöcyte.

The bacteroids continue to increase making a compact layer of unit thickness (Fig. 6). Then they pile up two or three thick, or more often they become arranged perpendicularly against the oöcyte membrane, giving the appearance in section, of an irregular pallisade (Fig. 7). The pallisade formation is usually complete in oöcytes of .09 by .11 mm. The layer becomes so compact that it buckles at both ends of the .11 by .4 mm. oöcytes, making ridges and knobs that project into the yolk (Fig. 8). During the course of the rapid growth of the egg from .6 mm. to 1 mm. in length, the bacteroid layer along the sides is stretched again to one or two bacteroids thick while the ridges at the ends enlarge and push farther into the yolk (Fig. 9). In the final more rapid growth period the lateral bacteroid layer is stretched more and is finally broken into small clumps which almost invariably lie in the angles between the ends of the follicle cells. The ridges of bacteroids at the poles of the 1 mm. oöcyte are principally broadened in this part of the growth period, forming at each pole an irregular disc-shaped mass approximately 15 μ thick at the center and 150 μ across.

If development of eggs is delayed by lack of copulation, dormancy of ovary during the non-reproductive stage, improper food, or by any other cause observed, the bacteroids do not become noticeably more

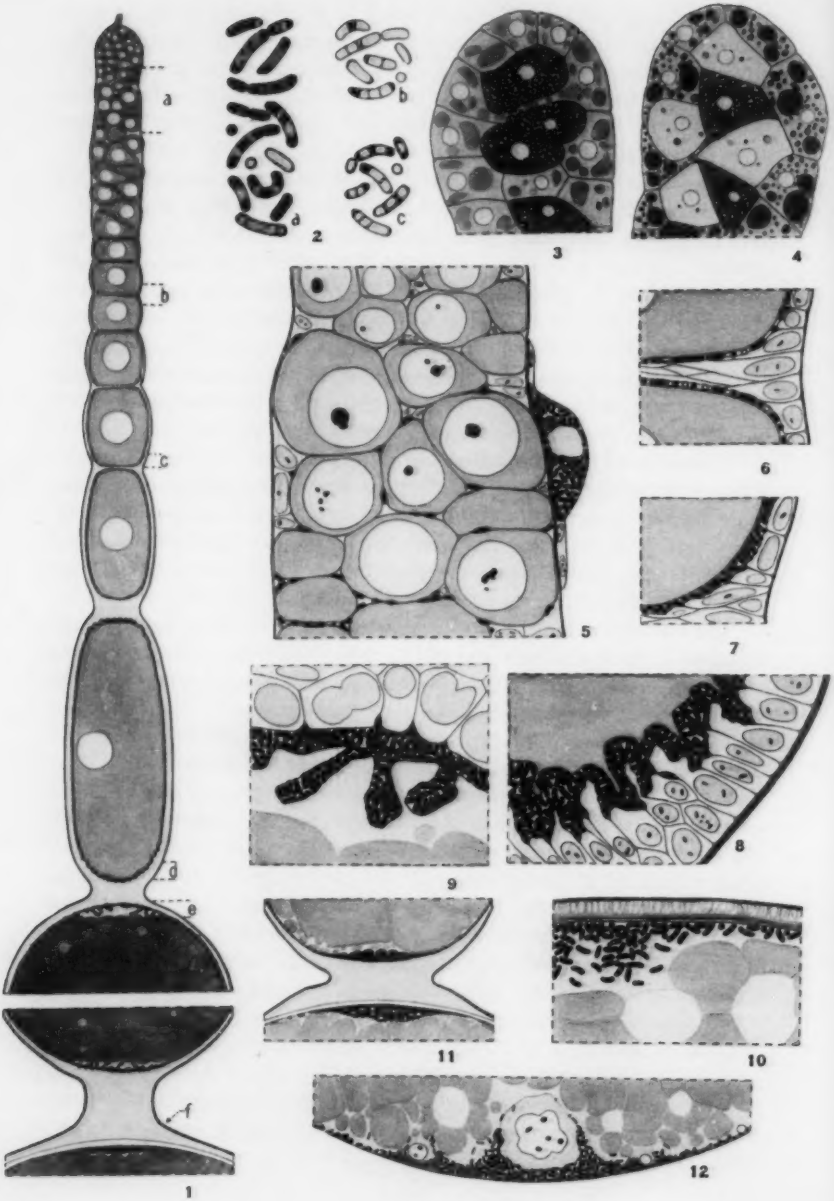

PLATE I

abundant than in eggs of healthy, normally fertilized reproductive females.

Throughout the ovarian history described, the bacteroids are definitely between the oöcyte membrane and the follicle cells. They have not been found within the follicle cells in any species, as described by Buchner (1912), although single bacteroids are often found isolated between the follicle cells (Fig. 8). These probably have become separated from the bacteroid layer since they are always found between the inner ends of the follicle cells. In many preparations the follicle cell

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Explanation of Plates

All figures were made from camera lucida sketches at the stated magnifications and reduced one-half in reproduction. Unless otherwise specified, the figures are from *Periplaneta americana* material: embryos fixed in Carnoy-Lebrun and stained with Giemsa; and other material fixed in Champy and stained with Haidenhain's iron hæmatoxylin.

For the sake of clearness and contrast, cellular details are omitted and the bacteroids depicted in solid black regardless of their appearance in the section. In the drawings of embryos, the areas occupied by bacteroids are heavily stippled.

Plate I

Explanation of figures

Fig. 1. A mature ovariole, schematized and parts of the large eggs omitted, showing the relative thickness of the bacteriod layer around the oöcytes. The lettered rulings at the side indicate positions from which Figs. 5 to 10 were taken. × 150.

Fig. 2. Typical bacteroids from around oöcytes, stained with Haidenhain's iron hæmatoxylin after fixation in (*A*) Champy, (*B*) Carnoy-Lebrun, and (*C*) Flemming. × 3,000.

Fig. 3. Typical fat body lobe from *Blatta orientalis*, Carnoy-Lebrun fixation. × 650.

Fig. 4. Fat body lobe with urate cells around the mycetocytes. × 650.

Fig. 5. Higher magnification of segment *a*, Fig. 1. Bacteroids around the distal oöcytes and in a mycetocyte at the side of the ovariole, outside the tunica propria. × 1,100.

Fig. 6. Higher magnification of segment *b*, Fig. 1. Bacteroids as a single layer between oöcytes and follicle cells. × 1,500.

Fig. 7. Higher magnification of segment *c*, Fig. 1. Beginning of pallisade formation. × 1,500.

Fig. 8. Higher magnification of segment *d*, Fig. 1. Bacteroid layer buckling at the end of .5 mm. oöcyte. × 1,500.

Fig. 9. Higher magnification of segment *e*, Fig. 1. Ridges of bacteroids at the end of a 1 mm. oöcyte, yet limited between oöcyte membrane and follicle cells. × 1,500.

Fig. 10. Higher magnification of segment *f*, Fig. 1. Bacteroids dispersing through the polar cytoplasm of a mature egg. × 1,500.

Fig. 11. Posterior end of 1 mm. oöcyte and anterior end of mature egg of *Blatta orientalis*. × 150.

Fig. 12. Bacteroid mass, with one giant nucleus and three smaller ones, at the posterior end of an egg after 60 hours incubation. × 650.

wall is very distinct both in well-fixed tissues and in shrunken material. The oöcyte membrane is very thin and is visible only as a definite limit to the cytoplasm.

Shortly before the egg is oviposited and soon after the first appearance of the chorion, the oöcyte membrane disappears and a new membrane, interpreted as the vitelline membrane, is formed between the bacteroids and the chorion (Fig. 10). The bacteroids, formerly limited to definite ridges, now disperse throughout the cytoplasm between the vitelline membrane and the yolk so that by 36 hours after oviposition there is a homogeneous disc-shaped mass of bacteroids at each pole, and numerous isolated clumps around the periphery of the egg. No bacteroids have ever been seen within yolk granules, as reported by Buchner (1912, his Fig. 6).

Buchner (1912) explained the polar concentrations by assuming an active migration of the bacteroids toward the poles. Such an assumption is not necessary if the differential rate of surface increase is considered. For example, an oöcyte 90 μ in diameter and 100 μ in length is surrounded by a uniform layer of bacteroids, while one 160 by 600 μ has very noticeable concentrations at the poles. Considering the oöcytes as cylinders, the ratio of end area to side area of the smaller is 6360:28,300 and of the larger is 20,100:301,600. This is an increase in end area of 201 per cent and in side area of 966 per cent; or, the side has expanded 4.8 times as much as the end. The original end of the oöcyte, however, enlarges at most to 140 μ, the remainder of the increase in diameter being accounted for by curving of the side, thus adding to the calculated growth of side surface. Consideration of the changing proportions during the final growth period is an even more convincing argument for this point. In enlarging from .35 by 1 mm. to 1 by 3 mm., the area of the sides increases approximately ten times while the original end of the egg expands less than 50 per cent. Without calculating the differential increases accurately, it is obvious that these differences are sufficient to account for the polar concentrations and the later thinning of the lateral layers, if the bacteroids multiply at the same rate all around the oöcyte.

In all species of roaches studied, the ovarian history of the bacteroids is remarkably uniform, the only differences being in the number of the bacteroids present and in the structure of the ovary itself.

Periplaneta australasiæ is like *Periplaneta americana* in all respects noted. *Eurycotis floridana* differs from these only in having smaller polar masses of bacteroids.

Blatta orientalis shows the same bacteroid relations around the smaller oöcytes, but the layer becomes much thicker, buckling distinctly

in .3 mm. eggs. The resulting ridges increase to such an extent that at each pole of a .5 mm. egg there is a nearly solid hemispherical mass which enlarges to a maximum of 60 by 75 μ in a .7 mm. egg, then spreads out in the final growth period into a vacuolated disc about 40 μ thick and 160 μ in diameter (Fig. 11). The vacuolated appearance is due to a partial restitution of the original ridges of the bacteroids. *Cryptocercus punctulatus,* although it has the same type of ovary as *Periplaneta americana,* ordinarily does not have bacteroids around the first four to ten distal oöcytes. The bacteroid layer becomes 4 μ to 5 μ thick, with no tendency to form a pallisade arrangement. The layer buckles uniformly around the .6 mm. egg, with only slight polar concentrations until the side layers are thinned in the final rapid growth period. No mature eggs of this species were studied. Consistently in the *Cryptocercus punctulatus* specimens examined, about one-tenth of the young oöcytes were infected and apparently destroyed by the bacteroids. They penetrated the oöcyte membrane, usually in very early stages, and increased within the yolk until what had been an oöcyte was a mass of bacteroids. A similar condition is occasionally found in *Blatta orientalis* and rarely in *Periplaneta americana.* In these latter, however, the bacteroids never increase as much as in *Cryptocercus.* In *Blattella germanica* and the species of *Parcoblatta* studied, the ovarioles are very slender near the germarium, each containing only one oöcyte in cross-section in contrast to the two, three or four in a similar section of a *Periplaneta* ovariole. Accompanied with this difference is the absence of any bacteroids around the first two to four distal oöcytes. The most anterior one to have bacteroids always has the greater number at the posterior end. There are always two or more oöcytes surrounded by bacteroids anterior to a completed follicle, which here, as in *Periplaneta,* splits the layer between oöcytes. The bacteroids gradually become more numerous, making a double layer or an incomplete pallisade around the oöcyte. There may be a slight buckling at the corners and at most a triple layer at the poles. In the final enlargement of the egg, the bacteroids become scattered around the sides while the end concentrations remain as thin layers.

In all species of roaches studied, bacteroids invariably occupy the space between the follicle cells and the oöcyte until the vitelline membrane begins to form, at which time they enter the egg cytoplasm.

After oviposition, while the early cleavage nuclei are dividing within the yolk, the bacteroids retain their position against the vitelline membrane. Many of the first nuclei to reach the periphery (two days incubation), enter portions of cytoplasm containing bacteroids, and within a few hours sink again into the yolk accompanied by the bacteroids.

These nuclei, with their cytoplasm and bacteroids, I shall call " primary mycetocytes." Others, indistinguishable from the above, come to the periphery and return into the yolk without bacteroids. These are the yolk nuclei or vitellophags. The remainder of the nuclei stay at the surface and form the blastoderm, which is continuous by the third day, except at the regions of the polar masses of bacteroids.

The course of events at the poles is not so simple as that for the rest of the egg surface. Each polar mass (Fig. 12) receives five to ten nuclei toward the end of the second day of incubation. The number increases to about forty by the fourth day. Most of them resemble the primary mycetocyte nuclei but a few (one to four) of the original ones become very large and have a considerable volume of clear cytoplasm. Apparently these giant nuclei do not divide and their later history is unknown except that they become separated from the bacteroids early in the migration of the polar masses. After this, they are not readily distinguishable from the ordinary vitellophags.

On the third day, the anterior bacteroid mass breaks into several smaller clumps which within two days sink into the yolk, reunite and proceed toward the center of the egg. The posterior bacteroid mass at

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PLATE II

Explanation of Figures

FIG. 13. Posterior bacteroid mass passing ventrally and anteriorly between the yolk and the embryonic rudiment. One giant nucleus has been left behind. Posterior ventral segment of a median sagittal section of an egg after 3½ days incubation. × 150.

FIG. 14. Bacteroid masses in the yolk. The outlines of the yolk granules are shown only around the bacteroid clusters. This and the following eggs have yolk granule and oil globule arrangement similar to that of Fig. 13. Median sagittal section of a six-day embryo. × 55.

FIG. 15. Bacteroid masses uniting near the center of the yolk to form the primary mycetome. Median sagittal section of a nine-day embryo. × 55.

FIG. 16. Eleven-day embryo with the primary mycetome still intact. × 55.

FIG. 17. Longitudinal section of the last thoracic segment and the anterior part of the abdomen of a 12-day embryo, nearly full length of the ovary. Approximately at the location of guide line *b* on Fig. 18. Many cells of the fat body contain bacteroids. × 110.

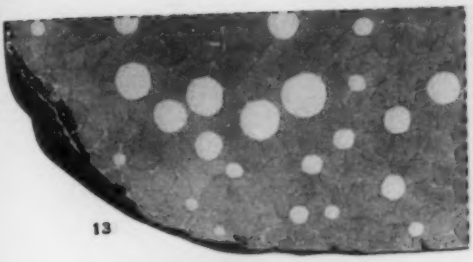
FIG. 18. Cross-section through the third abdominal segment of 12-day embryo (from the level of *a* on Fig. 17). × 110.

FIG. 19. *Parcoblatta pennsylvanica* embryo at approximately the same stage of development as that in Fig. 14. Bacteroids moving in small streamers between the yolk granules. × 70.

FIG. 20. *P. pennsylvanica* embryo intermediate in development between those of Figs. 15 and 16. Bacteroids in the primary mycetome. × 70.

FIG. 21. Portion of an ovariole of a four-month-old *Periplaneta americana*. Bacteroids are located in the mycetocyte outside the tunica propria and as a small clump between two oöcytes around which they are dispersing. × 1,000.

FIG. 22. Median section through the mycetome of a ten-day embryo. × 300.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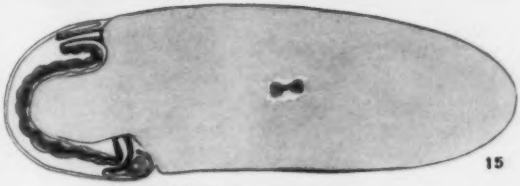
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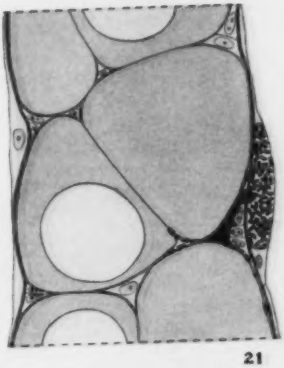
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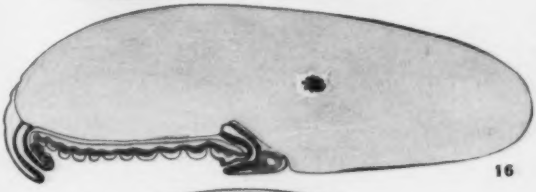
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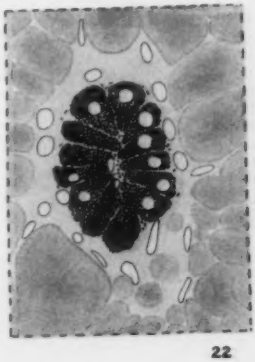
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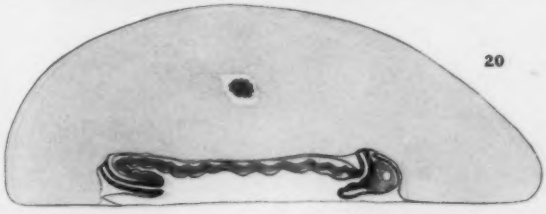
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PLATE II

this time is nearly spherical and lies at the posterior end of the newly differentiated embryonic rudiment. Near the end of the third day this mass begins to migrate anteriorly and ventrally, passing between the germ band and the yolk to about the middle of the embryo (Fig. 13), then it swings directly into the yolk (fifth day), either as a solid mass or as several clumps which reunite after proceeding a short distance. Both polar masses now move slowly toward the center of the yolk (Fig. 14) being joined on the way by the small lateral masses. All bacteroid masses join just anterior to the center of the egg about the end of the eighth day (Fig. 15) to form a compact mass approximately .1 mm. across, which mass Buchner (1912) called the "primary mycetome." The bacteroids within this mycetome are arranged in pyramidal segments, fitted together to form a sphere (Fig. 22). Each segment has one or two nuclei. Around the bacteroid sphere there is a single to double layer of vitellophag nuclei. No cell walls have been distinguished in this mass.

Heymons (1895) believed that the nuclei associated with the bacteroids increase by amitotic division. Although mitotic figures are rarely found and the nuclei are often lobulated as if pinching in two, I am not convinced that there are not enough mitotic divisions to account for the very slow increase in the number of these nuclei.

There is no question that the bacteroids do exhibit definite migrations. The posterior bacteroid mass in some eggs moves as much as 2.5 mm. in five days. This mass is made up of a loose clump of bacteroids and mycetocyte nuclei, surrounded by a layer of vitellophags. In some eggs the anterior surface of the mass is very irregular as if it were pushing forward between the yolk granules. In others, the yolk in the path over which the bacteroids have passed is more homogeneous than the surrounding yolk, suggesting that it had been dissolved. The small lateral masses unquestionably flow inward between the yolk granules in company with one or two ameboid nuclei. The bacteroids in all cases appear to be carried along passively with the mycetocytes and vitellophags.

So far as can be determined, all bacteroids in the egg are enclosed in the primary mycetome for about three days, after which some apparently leave the mycetome and migrate to the embryo.

At eleven days incubation the embryo has straightened out along the ventral surface of the egg and is approximately one-half the length of the egg (Fig. 16). Segmentation is complete on the ventral side, but the dorsal wall is formed only on the most posterior three or four abdominal segments. The lateral walls are formed dorsally to a line running diagonally around the egg from the head to the most anterior

point of the completed dorsal wall. The yolk is rapidly enclosed from this time on by dorsal and anterior growth of the lateral walls and elongation of the whole embryo so that by twelve days the dorsal wall is formed to the third abdominal segment (Fig. 17), and by fifteen days the embryo is as long as the egg and the yolk is entirely enclosed. At eleven days the fat bodies are rudimentary and strictly segmental, while the gonads are elongated masses of cells. By twelve days the gonads are definite cords, and by thirteen days the ovariole rudiments are recognizable as segmental enlargements of the ovary. The gut epithelium is continuous along the ventral side of the yolk between the stomodeum and the proctodeum and extends dorsally around the yolk as a discontinuous cell layer. The body cavity ends blindly at its anterior margin beyond the limits of the gut epithelium (Figs. 17, 18).

In the period between eleven and twelve days of incubation, the bacteroids within the mycetome lose their definite arrangement. The mycetome elongates and breaks and bacteroids appear between the yolk granules posterior to the mycetome. They also appear against the gut epithelium, in the body cavity, and in cells of the lateral lobes of the fat body bordering the body cavity. Immediately after the mycetome breaks, its posterior wall appears to have moved into the yolk. After eleven days of incubation, the number of bacteroids against the gut epithelium increases, reaching a maximum at about the eighteenth day. After that time they rapidly disappear from the gut, apparently by degeneration. The nuclei of the mycetome become indistinguishable from the vitellophags, and apparently most if not all of them fuse with the gut epithelium late in embryonic development to become the definitive entoderm, much as described by Stuart (1935) for a grasshopper.

The foregoing description of the primary mycetome, behavior of the bacteroids, and embryonic development in general, are matters of direct observations on fixed and stained material. Since it is impossible to see the bacteroids within the dense, living egg, their behavior must necessarily be interpreted from static material.

Heymons (1895) stated that the bacteroids move centrifugally from the primary mycetome through the wall of the gut and into certain cells of the fat bodies, after the gut completely encloses the yolk. Considering the stage of embryonic development and the places in which the bacteroids are found at the time, the following method of infection seems most probable. The vitellophags liquify the yolk around the primary mycetome which then loses its compactness. This liquid digestion product suddenly flows toward the embryo, separating the posterior cells of the mycetome. The nuclei are detained by the dense yolk granules, but some of the bacteroids are carried along passively in the

flow until they reach the embryo. If these bacteroids come in contact with the formed gut epithelium, they are retained while the liquid that carried them diffuses through. If they come in contact with the incomplete margin of the gut, or pass immediately anterior to the gut, they enter the body cavity directly. Within the cavity they are carried more posteriorly and ventrally and brought into contact with the fat bodies.

It seems possible, however, that in the early migration of the bacteroids a few may have remained with the embryonic rudiment while the rest proceeded to the primary mycetome.

Most, if not all, of the cells of the lateral lobes of the abdominal fat bodies bordering the body cavity take up a few bacteroids in the cytoplasm between the cell nucleus and the body cavity, thereby becoming mycetocytes. No differences between the cells of different parts of the fat bodies are discernible before infection, nor has any mechanical principle been found that would explain selective infection, yet the fact remains that neither the dorsal nor the ventral parts of the fat bodies become infected. As development proceeds, uninfected fat cells inclose the mycetocytes, or possibly as Buchner (1912) thinks, the infected cells sink into the fat body. The segmental fat bodies enlarge and fuse, having completely enclosed the gonads in the meantime. The bacteroids within each mycetocyte multiply rather slowly, causing an increase in mycetocyte size from approximately 12 μ at the time of infection to 25 μ at the time of hatching, and 30 μ at the first moult. Few mycetocytes get larger than 35 μ in diameter. The mycetocyte nuclei remain distinctly eccentric until two or three weeks after the roach hatches. No indication of mycetocyte division has been found, and no increase in numbers of mycetocytes in a roach is discernible after the first infection. The final form of the fat body is attained by the time of the second moult by two processes; dispersal of the mycetocytes among the ever enlarging fat cells before the roach hatches; and later separation of the mass into lobes of varying sizes, most of which have cores of mycetocytes.

Infection of the ovary has been considered to be a more complicated process than has that of the fat body. Buchner (1912) considers it probable that the bacteroids pass directly from the mycetocytes (Fig. 5 and Buchner, 1912, his Fig. 1), through the young follicle cells to the oöcytes. Fraenkel (1921) reported that she has seen steps in this process (her Figs. 1, 2). They do not specify, but intimate that this process is repeated for the infection of each oöcyte. It seems improbable, however, that a non-motile element could pass through the wall of the mycetocyte, across a potential cavity, through the very tough tunica propria and finally through the follicle cell.

The relationships between bacteroids and oöcytes described above (p. 439) exist in immature ovaries as young as four months (second moult). The ovaries of a young first moult roach are all like the tips of older ovarioles. Each ovariole is closely surrounded by mycetocytes outside the tunica propria. A cursory examination of the ovary at this stage reveals no bacteroids within the tunica. Before the second moult, a few oöcytes in the posterior part of each ovariole have enlarged to two or three times their original diameter. Against some of these, there are clumps of bacteroids apparently dispersing around the oöcytes (Fig. 21). These groups of bacteroids are often very close to mycetocytes from which they sometimes appear to be flowing, but no break in the tunica propria has been found.

Careful study of ovaries of roaches younger than three months almost invariably reveals the presence of one or more bacteroids in each ovariole, usually among the more posterior oöcytes. The bacteroids in this stage are easily overlooked because they stain much like the chromatin of the oöcyte nuclei. Either they occur in greater numbers or they are more easily recognized between the enlarging oöcytes of the three-month ovaries than between the small oöcytes of the one-month ovary.

In consideration of the observations just given, the following is the most probable method of infection. At the time of the fat body infection, a few bacteroids find their way into the mass of ovarian cells and remain there, increasing very slowly for a time. Within twenty-four hours the ovary becomes a compact cord of germ cells, between which are scattered the bacteroids. The ovarioles are formed as outgrowths from the original cord, enlarging at first entirely through multiplication of the oögonia. As this multiplication is greatest at the distal end, the bacteroids there become more scattered. The oögonia at the basal end of the ovariole soon cease division. Their chromatin passes through pachyphase before the roach hatches, and becomes diffuse soon after the first moult. With the advent of the diffuse chromatin the oöcyte begins more rapid growth and the bacteroids begin dividing and gradually spread over the surface of the oöcytes (Fig. 21). This dispersal is undoubtedly accelerated by the shifting of the oöcytes in the ovarioles and by pressure on the ovariole from general body movements. With the completion of the follicle around the most posterior oöcytes, the spread of bacteroids is limited to a forward migration which keeps pace with the development of new oöcytes to the infectable stage.

In review, the following observed facts favor this hypothesis of ovarian infection: (1) the ovary is a rough mass of cells without definite borders at the time of fat body infection; (2) there are a few

bacteroids in the very young ovary; (3) the first obvious infections are small clumps of bacteroids which appear to be spreading around the oöcytes. These clumps may be in the center of the ovariole, far removed from a mycetocyte; (4) passage of bacteroids from mycetocytes to ovarioles has not been convincingly demonstrated; (5) there are always several oöcytes with bacteroids at their surfaces anterior to a completed follicle; and (6) in ovarioles like those of *Periplaneta,* there are usually bacteroids around all oöcytes posterior to the tip, and so arranged as to give the impression that they had moved anteriorly in the angles between the oöcytes, while in ovarioles like those of *Parcoblatta* where each oöcyte fills the entire cross-section of the egg tube, the bacteroids are arranged evenly around the most anterior infected oöcyte.

The principal points against this hypothesis are: (1) the migrations of the bacteroids have not been seen in living roaches; (2) no bacteroids have been seen in the ovary for two weeks after the supposed time of infection (possibly due to lack of differential staining); and (3) the hypothesized retardation of bacteroid growth in the early ovary has not been established. This last objection, however, is apparently the same factor that limits the bacteroids to oöcytes that have passed a certain stage of development.

No differences have been found in the bacteroid behavior throughout the embryonic development of *Periplaneta americana, P. australasiæ* and *Eurycotis floridana.* However, *Blatta orientalis, Parcoblatta pennsylvanica, P. uhlerania,* and *P. latta* differ from *Periplaneta* in the effect of a median ventral rather than a posterior embryonic rudiment. In *Parcoblatta* the polar masses migrate directly into the yolk. In this migration, the bacteroids may be in small separated clumps, each with two to four nuclei, and pass close to the embryo (Fig. 19), or they may be in a large loose mass and pass deeper through the yolk. The lateral clumps join the general mass while en route but remain distinct until they reach the center of the egg. Here all masses unite to form a primary mycetome (Fig. 20) very similar to that of *Periplaneta americana.* In *Blatta orientalis* the polar masses are much larger, but the behavior is similar to that in *Parcoblatta.* Bacteroid behavior after formation of the primary mycetome is the same in all species studied, so far as could be determined. I have no embryological material of *Cryptocercus punctulatus* and so little of *Blattella germanica* that it warrants no comparisons nor conclusions as to bacteroid behavior. Buchner (1912) described a primary mycetome in the last-named species.

I wish to express my gratitude to Dr. Fernandus Payne and to the other members of the Faculty of the Zoölogy Department of Indiana University for their criticism and suggestions during the progress of this work.

Summary

1. The staining reactions, morphology and occurrence both intra- and intercellularly definitely preclude the intracellular bacteroids of the roaches from the category of mitochondria and link them closely to both the diphtheroids and the Rickettsia.

2. The number of bacteroids between the oöcyte membrane and the follicle cells increases until there is a uniform layer two or three bacteroids thick. By differential increase of the oöcyte surface this layer becomes thinner along the sides.

3. Before the egg is oviposited, the original oöcyte membrane breaks down and permits the bacteroids to enter the cytoplasm.

4. As the embryo develops, the bacteroids, accompanied by nuclei similar to the vitellophag nuclei, move in masses to the center of the yolk.

5. Later a few bacteroids from this central mass move posteriorly between yolk granules through the incomplete margins of the gut epithelium into the body cavity. From there most of them are taken up by the cells of the lateral lobes of the fat bodies while a few are caught between the cells of the forming ovaries.

6. The bacteroids within the ovariole seem to lie dormant for several weeks, then they multiply rapidly and spread over the surfaces of the enlarging oöcytes.

7. Newly enlarged oöcytes from the germarium are infected by bacteroids moving anteriorly from older oöcytes.

8. Bacteroid behavior varies between roach species with differences in number of bacteroids, in structure of the ovary, and in position of development of the embryonic rudiment.

9. All bacteroid migrations appear to be passive.

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AUTOGENOUS TRANSPLANTATIONS OF PIGMENTED AND UNPIGMENTED EAR SKIN IN GUINEA PIGS[1]

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The experiments to be described consist in a continuation of a series of investigations begun by Loeb and by Carnot and Deflandre, and studied further by Sale and Seelig and others, concerning the fate of autogenous transplants of pigmented and unpigmented skin in the ear of the guinea pig. Carnot and Deflandre (1896) and Loeb (1897) independently carried out experiments in which they transplanted pigmented and unpigmented epidermis from the ears of guinea pigs. In certain respects the results obtained by these investigators were similar, but in other respects differences developed. When unpigmented ear skin is autotransplanted to a defect in pigmented skin, the graft is either lost by scaling, or is invaded and replaced by pigmented epithelium from the surrounding epidermis. An autogenous graft of pigmented skin to a defect in a white area, however, persists; and in the course of time the pigmented cells grow out and invade the surrounding white epidermis, thus increasing the size of the colored graft. Although it is not especially stated, Loeb's transplants were autogenous, whereas Carnot and Deflandre state that they successfully carried out homoiogenous and even heterogenous grafts. The latter investigators furthermore report that while grafts from one species to another as a rule failed to grow, grafts from guinea pig to rabbit, and from rabbit to guinea pig, appeared to persist for a while, although with certain difficulties. These authors could not detect any differences in behavior between autogenous and homoiogenous transplants.

The investigations of Loeb were carried further by Sale (1913) and Seelig (1913), who compared autogenous and homoiogenous grafts of pigmented and unpigmented skin in guinea pigs. Sale showed that auto- and homoiogenous transplants of pigmented epithelium to white skin behave differently. The large majority of autogenous grafts survive and outgrowth of pigmented epidermis into the adjoining white

[1] These investigations were carried out with the aid of a grant for research in science made to Washington University by the Rockefeller Foundation.

epithelium is the rule. Only a few of the homoiogenous pigmented grafts remained alive, but in these cases invasion of the adjacent white epidermis did not occur. Instead, the pigment-producing function of the transplanted cells was injured and the graft gradually lost its pigment. As a rule an accumulation of lymphocytes underneath or in the transplanted epidermis preceded a partial separation of the transplant and its destruction in the majority of cases. Seelig has shown that unpigmented grafts when auto- or homoiotransplanted to defects in pigmented skin, are invaded and replaced by the adjacent pigmented epidermis.

Rand and Pierce (1932), studying autogenous and homoiogenous transplants of white ventral skin to pigmented areas in the tadpole, concluded from gross and microscopic observations that pigmentation of these grafts occurred through invasion and replacement of the graft by the surrounding epidermis. Barriers of hen's eggshell membrane could temporarily protect the graft from invasion and destruction. In autogenous grafts this invasion did not occur so regularly as in homoiotransplants. Herrick (1932) confirmed this observation so far as homoiogenous grafts in tadpoles were concerned, but concluded that autogenous grafts of unpigmented ventral skin retain their specificity and are not invaded for more than one hundred days.

Seevers and Spencer (1932) were unable to confirm the results of Loeb in regard to outgrowth of autogenous pigmented skin grafts into the surrounding white skin and the invasion of unpigmented grafts by surrounding pigmented epithelium in guinea pigs. Rectangular grafts having areas of about two square centimeters were made and the animals were observed for as long as nine months. During this time the transplanted black epidermis retained its pigment but it did not grow out into the neighboring white skin in any case. A few white grafts transplanted to defects in pigmented areas did become slightly darker along their borders after four to six months, but many such grafts remained unpigmented throughout the period of observation. In no case was the color of the hair changed, either in the graft or surrounding tissue. Except in the case of transplants to back and belly in agouti guinea pigs, there is no definite statement as to the part of the animal's body where these grafts were made; in the case of the experiments of Loeb and the other workers the ear was used without exception.

The technique used in our experiments was similar to that used by Sale and by Seelig. Guinea pigs were selected which had either one pigmented and one white ear, or sufficiently large pigmented and white areas on either one or both ears. With aseptic precautions a circular area of each type of skin approximately 4 mm. in diameter was re-

moved with a sharp razor. As little as possible of the connective tissue underlying the epidermis was included in the excised tissue. Bleeding, usually slight, was controlled by pressure. The skin pieces removed were placed in 0.9 per cent sodium chloride solution and cut to the desired size, usually about 2 mm. square. After oozing had ceased the grafts were placed on the denuded areas according to the experiments described, blotted, and alcohol-soaked cotton dressings were firmly applied, held in place with collodion. The dressings were usually loosened at the end of ten days, and subsequently observations and measurements were made at intervals of a few days or weeks for several months. Thirty-two animals in all were used and eighty-three individual grafts were made. Approximately one-half of the grafts did not survive, probably owing to errors in technique in our experimental procedure, or more often perhaps owing to scratching of the wound on the part of the animals.

The following data give a brief summary of the experiments performed and the survival of the grafts. It is interesting to note that practically the same proportion of pigmented and unpigmented grafts survived.

	Total	Survived
White grafts to pigmented skin	34	15
Single pigmented grafts to white skin	24	10
Double pigmented grafts to white skin	11	5
Triple pigmented grafts to white skin	1	1

In making the double and triple pigmented grafts a small area of intact white skin was usually left between the defects. Multiple transplants of white to pigmented skin were also made, but as they did not behave differently from single transplants, they are included in the discussion of the latter. The behavior of some of the representative grafts is described below.

Autogenous Transplants of Unpigmented Skin to Defects in Pigmented Skin

Our observations in fifteen cases where we were certain that the grafts were present were in accord with those of Loeb, Carnot and Deflandre, and Seelig. Complete invasion of the graft occurred in all cases. During invasion by surrounding pigmented epithelium there was, we also noticed, some scaling of the superficial layers of the graft. This appeared to persist as long as any white epidermis was yet visible, but its exact degree could not be determined grossly. In Seelig's experiments the time required for complete invasion varied from 13 to 90 days. We observed complete invasion in one case at 24 days, and in

the others the time required varied up to about ninety days or more. In one instance 200 days were required, and here the grafting was associated with considerable thickening of the underlying tissues, and this reaction, or perhaps the condition which gave rise to it, may have injured the host epidermis and thus have been responsible for the slow growth.

After complete invasion the site of the graft was usually somewhat thickened and appeared more deeply pigmented than the rest of the ear. Since similar observations have been made in connection with normal regeneration, this perhaps indicates that the presence of a graft of unpigmented epidermis does not greatly alter the normal regenerative processes of pigmented epidermis except in so far as rate of repair is concerned.

No correlation between the rate of invasion and the color or degree of pigmentation of the skin surrounding the transplant could be noted, grafts being made in defects of black, gray and brown skin.

Single Autogenous Transplants of Pigmented Skin to Defects in Unpigmented Skin

The results of these experiments likewise confirm the earlier observations of Loeb, Carnot and Deflandre, as well as those of Sale. Outgrowth of pigmented epidermis from the graft occurred in each of the ten cases studied. Some of these grafts were observed for 280 days. An average of about twenty days is required before a recognizable increase in the size of the pigmented area occurs. Occasionally the graft shriveled somewhat in this period, and in two cases seemed to have been lost. However, in these two cases a small point of pigment could be recognized at the center of the defect after 10 days and subsequently grew at a rapid and constant rate remarkably parallel to that of those which had remained intact during the first 20 days.

There appears to be a tendency for the outgrowth to be most rapid during the period between the twentieth and eightieth day following transplantation. During this time the diameter of the pigmented area increased from the original 2 mm. to a size somewhere between 4 and 6 mm., and hence it must have extended over the margins of the original defect in the skin. In most cases it was not possible grossly to determine whether the removal of the skin and subsequent regeneration in the area surrounding the transplant affected the outgrowth of the pigmented epidermis in this area. Since recognizable outgrowth did not occur until twenty days after grafting, regenerative processes of the epidermis and dermis over the denuded area had occurred by this time,

so that a definite demarcation between the original defect and the surrounding skin did no longer exist at this time.

After about eighty days the growth rate usually began to decrease, but the outgrowth continued at a decreasing rate for as long as two hundred and eighty days after grafting. In two cases where the pigmented areas were oval in shape, growth in the largest diameter ceased at about one hundred and sixty days, but the width of the area increased slightly during the following one hundred days of observation. In Sale's experiments no data were given concerning the duration of the period of outgrowth.

As far as the direction of the outgrowth is concerned, it was of a fairly uniform character, roughly symmetrically surrounding the original graft. However, the margins of the zone of outgrowth often presented a frayed appearance, due to fine pigmented processes which appeared to push ahead of the main outgrowth. These processes did not seem to be oriented in any particular way with reference to the adjacent blood vessels, scar tissue, or neighboring pigmented areas except in one case, described below, in which outgrowth was less rapid for a time in the direction toward a neighboring area of normal brown skin.

The depth of pigmentation in the zone of outgrowth was usually of the same degree as in the original skin used for transplantation; occasionally, however, it appeared somewhat lighter, possibly due to the fine processes mentioned, between which there were strips of non-pigmented skin. The color of the outgrowth always corresponded to that of the original graft, whether the latter was black, gray or brown. We were unable to find any definite correlation between rate of outgrowth and color of the grafted piece. With one exception described below, all appeared to grow equally well. One of the best growing cases was a graft of gray epidermis, which had increased in size between the final two observations.

In this series we were not specifically concerned with the problem as to the possible effect of the amount of pigmentation or of the lack of it in the whole animal on the rate of outgrowth from the graft. In the majority of the animals used the area of pigmented skin was larger than the area of white skin, taking the body as a whole, but in one instance we used an animal that was almost entirely white except for one ear, which had a rather light brown color. From the skin of this ear two grafts were made to the white ear of the same guinea pig, and it was found that the diameters of the grafted areas increased 2 and 3 mm. respectively during a period of two hundred and forty days; whereas the average increase for the rest of the animals observed during this length of time was about 6 mm. In this single instance our results

appear to confirm, perhaps, those of Carnot and Deflandre, who found that pigmented grafts extend more rapidly on animals showing a higher degree of total pigmentation. In our animals, however, the pigmentation of the one brown ear was not intense, a fact which in itself may be sufficient to explain the slow outgrowth in this case, rather than the deficiency in total body pigmentation.

Where hair was present in the area into which pigmented epidermis extended, it remained unpigmented during the period of observation. This would indicate that the pigmented skin invades surface epithelium only and does not readily extend into the hair follicles. While we concede the possibility that this may ultimately occur, it evidently requires a longer period of time than that during which our transplants were under observation.

Multiple Autogenous Transplants of Pigmented Skin to Defects in Unpigmented Skin

Double and triple grafts were made in order to obtain additional proof that the original white skin surrounding the defect becomes invaded by pigmented epithelium of the graft. When two pigmented grafts separated by an intervening area of normal white skin become connected, it is necessary to conclude that this white skin must have been invaded by the pigmented epidermis. Five double transplants and one triple transplant were made. They were separated in most instances by intact white skin varying in width from 1 to 4 mm.; but in one case both grafts were placed 2 mm. apart in the same defect.

The double grafts behaved exactly like the single ones, in that outgrowth of pigmented epidermis occurred. In two cases, where the grafts were fairly close together, fusion occurred during the period of observation. In one of these, in which the grafts were separated by 1 mm. of intact white skin, they had grown together after thirty-seven days, forming an elongated dark patch from which processes extended radially to the margin of the ear on one side, and medially to a neighboring area of normal black skin. In the case of the triple transplant two of the pigmented areas, both in the same defect, had joined by thirty days; and by sixty days all three grafts had fused across 2 mm. of intervening white skin to form a single lobated area of black epidermis. In cases where fusion did not occur the grafts had been either placed too far apart for fusion during the period of observation, or, in one case already described, the rate of outgrowth was unusually slow.

In addition to the fusion of double transplants, invasion of normal pigmented skin by outgrowth from grafted skin was also observed. In one case already mentioned where the black graft was placed at a

distance of about 2.5 mm. from a normal area of brown skin, outgrowth occurred toward this brown area, although less rapidly than away from it. The outgrowth met the autochthonous pigmented margin after 95 days, and by 200 days had definitely invaded it for a distance of 2 mm. along a border of 8 mm. The black margin of the outgrowth could be clearly seen extending into the lighter brown skin. Such an observation was possible because of the difference in the character of the pigment in the autochthonous and in the transplanted area. It would have escaped detection if grafts indistinguishable in pigmentation became connected, or when a black graft joined normal black epithelium, as occurred in two cases.

In another experiment two defects, 6 mm. apart, were made in a wedge-shaped strip of white skin separating two large gray-brown areas. The smaller medial defect reached the pigmented skin on either side, while a very narrow margin of white skin was left on either side of the lateral defect. In the lateral defect a small piece of pigmented skin was placed, while the medial defect was left to heal without a graft. After a period of 56 days had passed the medial ungrafted defect was filled in by pigmented epidermis, and during a period of 74 days the outgrowth from the small graft in the lateral defect had extended across the two narrow white margins on either side. Accurate measurements of this original white zone were not made, but later observations at least indicated that the whole zone was being invaded from the adjacent pigmented surfaces, a process which was most marked along the lateral margin of the ear, at a distance of several millimeters from the graft. This suggests that in some cases the normal balance between pigmented and unpigmented epidermis may be lost. The character of the pigmented epithelium of this ear was somewhat different from that of the other animals used, being a rather uneven gray-brown, not unlike the margins of some of the outgrowths of grafted brown skin.

Discussion

We notice following transplantations a change in the equilibrium between adjoining types of epidermis which differ in their pigmentation. When, under the influence of such tissue disturbances, a shift in the balance between two neighboring types of epidermis takes place, the pigmented tissue seems to dominate over the unpigmented. However, we found indications that such an imbalance may also occur between neighboring types of epidermis which are both pigmented, but in which the kind and quantity of the pigment differs. It is conceivable that changes similar to those we have described may take place even between two adjacent areas of black skin, but such changes would not

become manifest. In our case we observed movements in autochthonous epidermis under the influence of a wound in close proximity to this skin. As to the causes of these movements in epidermis, they must be distinguished from those of ordinary regeneration in which the growth takes place into a defect. Apparently the equilibrium between different types of epidermis is a very labile one which can readily be disturbed by various interferences.

Summary and Conclusions

If white skin is autotransplanted into a defect in pigmented skin in the ear of the guinea pig, the pigmented epidermis gradually invades the white. At the same time some scaling takes place in the white skin, and in the end the white epithelium becomes replaced by pigmented epithelium. This process could be completed in a minimum of twenty-four days, but in other cases it took as long as two hundred days. In the end the area where the white epidermis was replaced by pigmented epidermis was thickened and showed deeper pigmentation. There was no difference noticeable in a limited number of cases in the rapidity of invasion of the white transplant by black, gray, or brown epidermis. Rate of invasion does not therefore seem to depend on the intensity or kind of pigmentation of the surrounding host epidermis.

If pigmented skin is autotransplanted into a defect in white skin in the ear of the guinea pig, an outgrowth of the pigmented epidermis into white autochthonous skin takes place. As an initial process pigmented as well as white epithelium grows into the defect, but this is followed by the growth of pigmented into the original white skin. Definite outgrowth was first observed at about twenty days following transplantation and the outgrowth became most rapid between twenty and eighty days. Following this period outgrowth usually continued at a decreasing rate but could still be observed as late as after two hundred and seventy days. The original hair of the invaded white epidermis remained unpigmented during this process, indicating that the pigmented cells invaded the surface epithelium first and had not yet penetrated into the hair follicle epithelium at the time when the experiments were concluded. There was no correlation observed between color and intensity of pigmentation of the transplanted epidermis and the rapidity of its outgrowth into the surrounding white skin.

In case multiple transplantations were made, it could be observed that the two neighboring pigmented transplants gradually invaded the white skin separating them and thus a junction between the two pigmented transplants was accomplished. This observation provides an

additional proof for the conclusion that it is actually the original white host epithelium which is invaded and replaced by the transplanted pigmented epithelium.

In accordance with these findings, we could observe a junction taking place between a black transplant and an area of brown autochthonous epidermis from which the transplant had originally been separated by an adjoining area of white skin. In this case it could be noted that the black skin invaded not only the white epidermis, but also the brown pigmented epidermis; and it may therefore be concluded that not only white skin may be invaded by black pigmented skin, but also pigmented skin may be so invaded.

In one case it could be observed that originally white skin became partly invaded by adjoining pigmented skin of the host. Thus the pigment pattern of the skin became changed, apparently spontaneously. However, it is possible that transplantation of black skin into white skin at a short distance from the area where the first-named change took place may have been responsible for this disturbance in the equilibrium between adjoining pigmented and unpigmented autochthonous epithelium.

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RECOVERY CHANGES IN TRANSPLANTED ANTERIOR PITUITARY CELLS STRATIFIED IN THE ULTRA-CENTRIFUGE[1]

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With the thought of possibly getting some light on the beginnings of malignancy, by upsetting the normal conditions within cells and observing the subsequent effects if any on the growth and functions of such cells, the authors subjected various rat tissues to intense centrifugalization and then implanted them in young rats. Inasmuch as they have been particularly interested in a type of vacuolation that appears in the basophile cells of the anterior pituitary body following the implantation of carcinoma (Guyer and Claus, 1932, 1933, 1934, 1935), they devoted special attention to the transplants of centrifuged pituitaries, and the present paper is a report of their observations on this material.

To avoid complications which might arise from the use of anæsthetics the 39 young adult rats from which the pituitaries were to be taken were killed by a blow on the head, the glands were rapidly dissected out and placed in isotonic Locke's solution. A small bit of each pituitary was used as a normal implant into a control rat, or in a few cases, for sectioning; the remainder was placed in the isotonic solution in the metal rotor of a Beams air-driven ultracentrifuge and rotated at a speed which produced a displacement pull of about 400,000 times that of gravity. The pituitaries from 20 rats were rotated for 20 minutes, then removed from the rotor, a bit was retained for cytological inspection and the rest was immediately implanted subcutaneously into twenty young rats of between 65 and 90 grams weight, one piece of pituitary to each rat. In a later experiment the pituitaries from 19 rats were removed and centrifuged, using exactly the same procedure as in the earlier experiment except that the material was centrifuged an hour before implanting it in the young rats.

The effect of an hour of such centrifugalization is shown in Fig. 5. The materials of the cell are almost wholly stratified. After 20 minutes of rotation in the centrifuge the stratification is very evident but not complete. As a result of such centrifuging the Golgi apparatus

[1] These investigations were supported in part by a Brittingham research grant and in part by a grant from the Wisconsin Alumni Research Foundation.

becomes compacted into what seems to be a single liquid mass and comes to occupy the centripetal side of the cell; that is, it is among the lightest of the cell contents. The nucleus is displaced toward the opposite or centrifugal side of the cell.

In basophile cells the nucleus seemed generally to be forced up against the cell wall but in the oxyphile cells a layer of deeply staining oxyphile granules often intervened between them. The oxyphile granules, indeed, seemed to be the heaviest particles in the cell and after prolonged rotation they became massed at the centrifugal side of the cell with the nucleus forced into their midst on the side toward the cell center.

Examination of material rotated for 20 minutes shows, however, that the displacement of different cell contents is at different rates of speed and that stratification in one kind of cell may be induced much more rapidly than in another, thus indicating different degrees of viscosity. The oxyphile cells of the anterior pituitary, for example, respond more slowly than do the basophiles and with the less prolonged centrifugalization one gets the impression at first that the nucleus is coming to lie at the centripetal side of the cell. This condition is temporary, however, and is probably due to the fact that the heavier oxyphile granules are being concentrated into the center and opposite side of the cell, temporarily displacing the nucleus toward the centripetal side. Eventually it is forced to the centrifugal side and, as just described, becomes more or less imbedded in the mass of oxyphile granules.

From their respective behaviors under centrifuging it is evident that the oxyphile granules found in pituitary cells are heavier than the basophile granules. The centrifuge also reveals that even in the large so-called basophile cells there are considerable numbers of oxyphile granules, since in such cells when differentially stained a thin zone of acid-stained particles is visible.

The chromatin content of the nucleus is densely massed at the centrifugal side although strands of an achromatic substance, presumably linin, remain stretched across the nuclear body, giving the appearance of being attached to the opposite nuclear wall. Evidence of this is seen in the nuclei shown in Fig. 5. Inasmuch as this condition has been rather fully discussed and depicted in a recent paper by Dornfeld (1936) in connection with cells of the adrenal gland, based in part on material from the same rats as were used by us in the present study, further comment on the nature of the nuclear displacements is unnecessary; the conditions seem to be similar in all respects.

The implanted tissue which had been centrifuged for 20 minutes

was removed and fixed for cytological study after different periods of growth, as follows:

Tissue planted		24	hours,	from	1 rat.
"	"	48	"	"	1 rat.
"	"	4	days,	"	1 rat.
"	"	7	"	"	4 rats.
"	"	14	"	"	5 rats.
"	"	21	"	"	4 rats.
"	"	28	"	"	1 rat.
Implants did not grow in					3 rats.
					20 rats.

The most striking fact to be noted in the transplanted pituitary cells was the rapidity with which those which survived resumed relatively normal appearance. At the end of 24 and of 48 hours respectively the removed implants showed the presence of much necrotic tissue and a heavy invasion of polymorphs, but this was true of control transplants as well; hence it cannot be attributed to the centrifuging. As soon as the vascularization of the transplanted tissue became well established this condition rapidly cleared up and the cells, particularly those near blood vessels, began to look normal. The last thing to be restored to its characteristic normal appearance was the Golgi apparatus. This structure seems to be very sensitive to changes in general, for even in normal, non-centrifuged anterior lobe material it tended to become clumped and pyknotic looking. The return of the nucleus to the central region of the cell and the redistribution of the various cell granules seems to follow promptly the cessation of centrifuging. In another set of experiments (ms. in press) carried on in this laboratory by Halcyon W. Hellbaum on centrifuged snake thyroid, the brief interval between the start of tissue fixation and its completion seemed to be sufficient for the nucleus to make an appreciable return toward the cell center; for with centrifuged thyroid tissues, in the halves of thyroid glands that were fixed in rapidly penetrating fluid such as Bouin's, the nucleus was noticeably nearer the centrifugal margin of the cell than in the other halves of the same glands fixed in more slowly penetrating fluids such as Champy's (bichromate-osmic-chromic acid) mixture. This would indicate that cytoplasmic elasticity enters as a significant factor.

Immediately and for some time after centrifuging, the Golgi complex seems to be but a dense rounded drop of material at the centripetal side of the cell. It comes back to its network-like configuration very slowly, attaining it again only at the end of two or three weeks. It first loses its dense appearance by breaking up into a multitude of fine droplets, so small often as to give almost a powdery appearance. As time goes on the droplets become larger, apparently

by more or less coalescence. In pituitary tissue which had been centrifuged for 20 minutes and then removed after an implantation of 14 days, the Golgi complex is usually back in the neighborhood of the nucleus though it is commonly still in a divided state of droplet-like particles. These are grouped more on one side of the nucleus although they may more or less surround it. A few examples of a return to the network type of Golgi body are to be seen in some of the basophile cells; still more occur in the oxyphiles.

Even at the end of three weeks a considerable amount of granular Golgi material is still in evidence although the prevailing Golgi apparatus is of the network type. However, this slow return of the Golgi complex to the condition of a network is probably not determined by the fact of earlier centrifugalization since control, non-centrifuged pituitary recovered after three weeks of transplantation may similarly show a considerable degree of granulation.

By the end of the fourth week the transplants, both centrifuged and non-centrifuged, seem to be rather generally on the decline, and possibly final disintegration is approaching. This is particularly true of the basophile cells; their Golgi material shows much granulation or droplet formation.

Figure 6 is a composite picture of two sections of recovered anterior pituitary which had been centrifuged for 20 minutes and then implanted for seven days. That the tissue is anterior pituitary gland is evident. Certain of the basophiles (*b*) and the oxyphiles (*o*) have been lettered to identify them more readily.

Of the 19 rat pituitaries which were centrifuged for an hour before implanting, only 2 survived. Operations at the end of two weeks revealed that 2 had been replaced by pus sacks and the remaining 15 had been resorbed. In the 2 bits of tissue which were alive at the end of two weeks the basophiles and oxyphiles seemed to be in a thriving condition. They did not differ in any noticeable particular from similar implants of the 20-minute series.

In one of the anterior pituitaries which had been centrifuged 20 minutes and then recovered after three weeks of implantation, a peculiar pituitary cyst was encountered (Figs. 3, 4). It strongly suggested the so-called colloid follicles so characteristic of the thyroid. Cysts of supposedly colloid-secreting cells are occasionally found in the pituitary glands of normal animals including man. The striking thing about the cyst in our transplant was the fact that it had apparently been stimulated to increased activity. The lumen was filled with a vacuolated, gluey looking mass and the secreting cells had become columnar instead of cuboidal, much enlarged, and in many cases they looked like goblet cells. This raised the question of whether

they were typical colloid cells or were mucus-secreting cells, and also of just what the difference between a colloid and a mucous secretion is. With the Mallory triple stain the secretion gave the typical blue colloid picture but when Mayer's muci-carmine stain was used the picture was just as typically that of mucus-secreting cells with various of the cyst-wall cells staining as deeply as do characteristic goblet cells. This was true even in sections which had been stained in Mallory's triple stain, and later destained, and stained again in Mayer's muci-carmine. Thyroid follicles, however, subjected to the same treatment did not give the muci-carmine response. The pituitary cyst from a control animal (Figs. 1 and 2), on the other hand, did give the typical muci-carmine reaction.

In Figs. 1 and 2 photographs of a cyst from the anterior pituitary of a control and supposedly normal rat are shown at different magnifications. While the contents of the cyst lumens seem the same in the control and in the experimental animals, the secreting cells are very different in appearance in the two. Those from the control (Figs. 1 and 2) are cuboidal, those from the centrifuged and transplanted pituitary are conspicuously columnar in appearance. Both sets are ciliated. We have thoroughly canvassed the possibility that such appearances as those shown in our photographs (Figs. 1 to 4) might be due merely to striations in the mucin or in the shrinkage space between the mucoid mass and the epithelium lining, but careful inspection of these and other adjoining sections, both by ourselves and others in our laboratory accustomed to making cytological observation under high

EXPLANATION OF PLATE I

1. Photomicrograph of a colloid or mucoid cyst in the anterior pituitary of a normal rat, showing the ciliated epithelial lining of the cyst wall and the contents of the lumen. × 376.

2. Photomicrograph of a part of the foregoing cyst, under higher magnification. The cuboidal, ciliated epithelial cells are obvious. × 848.

3. Photomicrograph of a mucoid cyst in anterior pituitary material that had been centrifuged in an ultracentrifuge for 20 minutes, then grown in a young rat for three weeks, after which it was removed and sectioned. The epithelium which lines the cyst wall consists of very active, columnar cells, mostly ciliated, though some have the appearance of being goblet cells. × 376.

4. Photomicrograph of a part of the cyst shown in Fig. 3. Occasional smaller cells are observable between the bases of the columnar cells and the basement membrane. × 848.

5. Photomicrograph of cells from a rat hypophysis centrifuged in an ultracentrifuge for one hour. The deeply stained material of the nuclei is the chromatin substance forced to the centrifugal side of the nucleus. In some of the nuclei strands of achromatic material, presumably linin, are visible stretching across the nuclear body, because still attached, seemingly, to the opposite nuclear wall. × 848.

6. Photomicrographs from two slightly different regions of a bit of anterior pituitary gland grown 7 days in a young rat after having been centrifuged for 20 minutes. Typical basophile (*b*) and oxyphile (*o*) cells are to be seen. × 848.

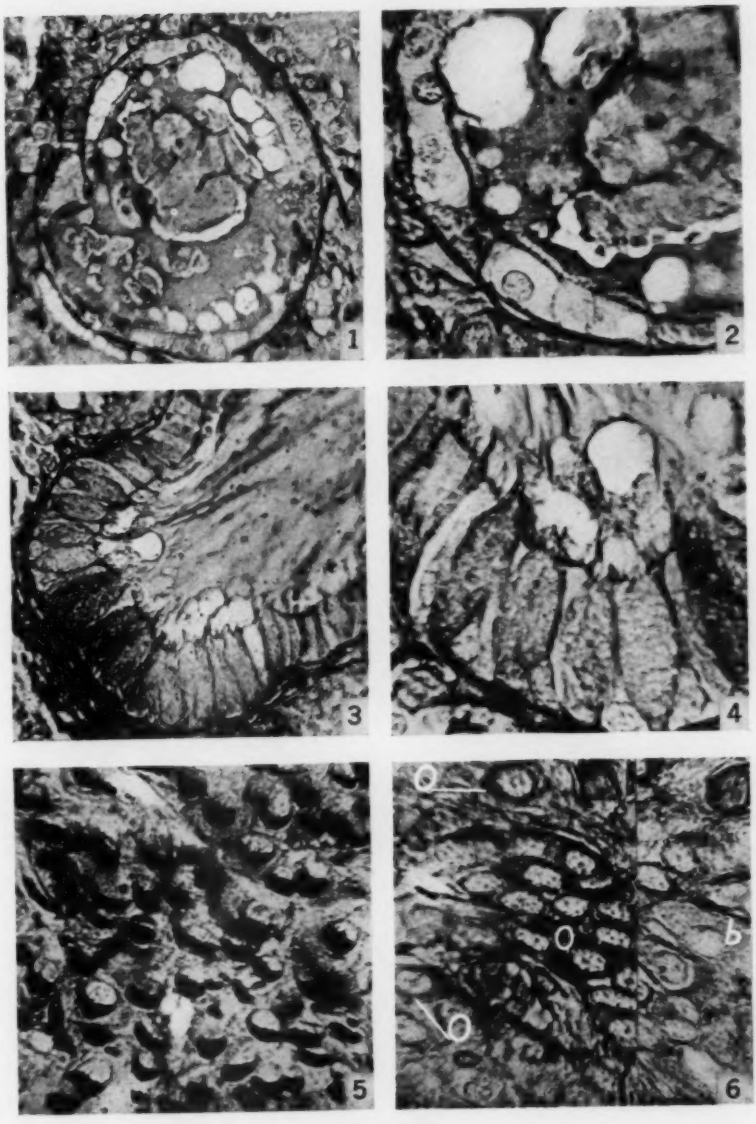

PLATE I

powered lenses, convinces us that the objects in question are not artefacts but cilia.

Whether the goblet-like cells of the transplant are transformed ciliated cells we are unable to say. And whether or not the wall cells of these cyst formations are to be regarded as unusual differentiations of pituitary tissue, as residual cells from an earlier epithelium, or migrant elements from the naso-pharyngeal region, we cannot say. The thing of chief interest in our present study is the enhanced secretory activity shown by the centrifuged cells.

In the literature dealing with such hypophyseal cysts the nearest approach to the types of cell shown in our preparations is that depicted by Rasmussen (1929) from sections of human hypophyses. Several of his pictures from human material look almost exactly like what we find in implanted centrifuged pituitary tissue from rats.

Summary

Anterior pituitary tissue which has had its cell contents stratified through 20 to 60 minutes of rotation in an ultra-centrifuge with a displacement pull of about 400,000 times that of gravity, returns in many instances to normal appearance, displaying the characteristic basophile and oxyphile cells, when transplanted into young rats. An hour seems to be nearly the maximum time such tissues can be thus rotated and remain viable. Nuclei forced completely to one side of the cell rapidly resume their normal location near the cell center, with their displaced chromatin contents apparently restored to normal distribution. The Golgi apparatus, concentrated by the rotation into a liquid drop at the centripetal side of the cell, is the last part of the cell complex to resume its characteristic appearance, which is that of a network. A mucoid cyst with hypertrophied and extremely active epithelial wall cells, most of them ciliated, is described and compared with the type of cyst that is occasionally found in the normal pituitary.

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COMPONENTS OF THE MITOTIC SPINDLE WITH ESPECIAL REFERENCE TO THE CHROMOSOMAL AND INTERZONAL FIBERS IN THE ACRIDIDÆ

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Introduction

While my own studies are concerned with specific components of the mitotic spindle, I believe the structure should be considered as a whole and I shall endeavor, accordingly, to summarize our knowledge of certain points.[2]

Centrioles

The beautiful demonstration of these structures in pole cells of *Drosophila* eggs which complete their development (Huettner and Rabinowitz, method published in 1933) must validate the reports of these structures in fixed material even for the skeptical. The following conclusions in regard to them seem to be warranted by careful work.

Physical Characteristics.—They are visible in living cells where they undergo vibratory motion and give evidence of being more rigid than the surrounding cytoplasm. They are usually spherical but their ability to vary in shape is well shown in the tree-crickets (Johnson, 1931) where the centrioles are minute spheres in the spermatogonia, comparatively large V's in the first spermatocytes and specialized rods in the second spermatocytes. In the protozoan, *Barbulanympha,* as shown by Cleveland et al. (1934), they are elongate, flexible rods of relatively large size. In all cases, they stain with iron-hæmatoxylin but in *Ascaris,* at least, they do not give the Feulgen nucleal reaction (Carothers, unpublished).

Position.—Centrioles are located usually just outside the nuclear membrane but they may be near the cell membrane, as in the first spermatocytes of the tree-crickets, or intranuclear, as in certain of

[1] This work was begun at the Zoölogical Laboratory of the University of Pennsylvania.

[2] A much fuller discussion of this subject is to be found in the third edition of E. B. Wilson's excellent book, "The Cell in Development and Heredity." Macmillan, 1925.

the Protista and in the male germ cells of *Ascaris*. During mitosis they come to lie on opposite sides of the nucleus, 180 degrees apart.

Origin.—Normally, they are self-perpetuating structures which undergo division at each mitosis. In addition, a so-called de novo origin seems to have been demonstrated in certain instances. Wilson (1901) showed that in artificial parthenogenesis in sea-urchin eggs numerous cytasters each with a centriole-like body arise in one egg. These "central bodies" and their cytasters divide and give rise to typical amphiasters which later are separated by cleavage furrows. Even more impressive is the fact that cytasters may unite with a nuclear aster to form a multipolar spindle with irregular distribution of the chromosomes to all poles followed by multipolar cleavage. A "de novo" origin may occur also in partheno-produced grasshoppers (Slifer and King, unpublished). Centrioles appear to be lacking during the maturation divisions of the eggs, yet the early cleavage cells of embryos arising from unfertilized eggs have typical centrioles. Here, however, there is the possibility of the survival of the egg centriole even if it does not function during meiosis. In the case of artificial parthenogenesis I would suggest that certain of the formed components of the cytoplasm may be able under especial circumstances to take on the function of centrioles.

Distribution.—Centrioles are present generally in the Metazoa. An exception is the oöcytes in which they may either be lacking as in *Ascaris* or present as in *Crepidula* where Conklin (1902) showed that a division center is formed with each pronucleus. They are lacking in the higher plants, while in the lower plants (cycads, *Ginkgo*, bryophytes and pteridophytes) they are absent in the somatic cells and those of the early germ-line but appear as typical centrioles with asters at a definite stage in the life cycle, namely, the last divisions which give rise to the male sex-cells.

Function.—Aside from their prominent part as division centers in mitosis, centrioles are associated with motility as the blepharoplasts which form the locomotor apparatus of the sperm cells of the lower plants, the flagellate Protozoa and the flagellate cells of sponges as well as the sperm cells of animals. In the case of *Barbulanympha*, referred to previously, and spermatids, the centriole divides and the proximal one takes no part in forming the locomotor apparatus. This suggests, as has been pointed out by other workers, that centrioles may contain two components only one of which functions as a blepharoplast.

Centrosomes

This term denotes the regions of specialized cytoplasm surrounding the centrioles about which the constituents are arranged concentrically.

Near the centrioles the groundwork is homogeneous or finely granular, and, in well-fixed material, the astral rays are seen to traverse it. Centrosomes vary greatly in size and complexity in different organisms and may be entirely absent as in some of the hypermastigotes (Cleveland et al., 1934).

Astral Radiations and Central or Continuous Spindle Fibers

Perhaps the most significant work to date on these structures is that just mentioned of Cleveland and his collaborators. They report, on what they consider absolutely conclusive evidence derived from both living and fixed material, that astral rays and central spindle fibers arise from the centrioles. Furthermore, and even more important, they state: "The fibers of the achromatic figure . . . arise from the opposite ends of the same organelles (centrioles [3]) as the flagella; and they are like flagella in individuality, size, and appearance both in living and stained organisms; but, unlike flagella, they are not able to move independently." Flagellates in roaches undergoing ecdysis form intracytoplasmic bundles of flagella; these are used as a basis of comparison. Lucas (1932) described a ciliature anlagen for *Cyathodinium* in which the cilia are formed intracytoplasmically, before they are evaginated upon the surface. These two cases show clearly that definite, thread-like structures possessing elasticity and tenacity can be differentiated in the cytoplasm; hence, there is little reason to doubt that spindle fibers exist in living cells essentially as they appear in well-fixed material.

Another well-established and critical fact, already mentioned, is that compound achromatic figures may be formed by the union of asters of different origin, due either to double fertilization where the two sperm may come from different males or to artificial fertilization, where cytasters may join with a normal amphiaster. Incidentally, tripolar spindles are the strongest evidence against the idea that the mitotic figure is due to a polarized field since lines of force form only between plus and minus poles. Another fact which does not fit with such a hypothesis is that in normal mitosis the rays from the two asters cross at the equator, whereas lines of force in a bipolar field are continuous from pole to pole. Both of these facts were recognized by early workers. I mention them now because several recent observers ignore them.

Interzonal Fibers

These are the fibers which connect the distal ends of daughter chromosomes for a brief time in anaphase. (See p. 482.)

[3] The parenthetical expression is mine.

Chromosomal Fibers (Half-spindle Fibers)

These structures pass from a fixed locus on each chromatid to the poles. The shape of the chromosome is correlated with the position of these loci.

Mitochondria

Perhaps these elements should be mentioned since they surround the spindle densely, especially in insect meiosis (Bowen, 1920), and probably prevent the fibers themselves from being seen in living cells.

Technique and Nomenclature

The material was fixed in either strong Flemming or my own fixative, the formula for which may be found in Dr. McClung's *Microscopical Technique*. The stains used were Flemming's tri-color and Heidenhain's iron-hæmatoxylin. Most of the material was stained prior to 1920.

In regard to terminology, I shall use the phrases "chromosomal fibers" or "chromosomal processes" interchangeably to designate the structures which pass from the chromosomes to the poles in preference to the expression "half spindle fibers" recently revived by Schrader, and I hope to show that the term "point of fiber insertion" should be changed to "point of origin."

Observations

My observations concern, chiefly, the chromosomal and interzonal fibers. The X-chromosomes of short-horned grasshoppers lend themselves very readily to the study of chromosomal fibers for two reasons:

1. They are a step in advance of the other chromosomes in the first spermatocytes. Consequently, they reach the physical condition of euchromosomes in metaphase at late diakinesis and at the actual metaphase are becoming diffuse for the following telophase.

2. The point of origin of spindle fibers varies in different species but is constant, typically, for the individuals of a given species; occasional exceptions to this rule occur, however. For example, most species have telomitic X-chromosomes but certain species of *Trimerotropis* and allied genera have atelomitic X-chromosomes with rare individuals which not only have both types but intermediate forms as well. Plate I, Fig. 1 shows partial complexes from three adjoining first spermatocyte metaphases of such an individual of *T. coquilletti*. At

C the X-chromosome (solid black) is telomitic, at *B* it is atelomitic with a process from each chromatid while at *A*, obviously, it is in a dilemma. The process for a terminal fiber is present, at the lower right end, the median processes are evident above, and in addition the opposite end has two subterminals going to opposite poles. The chromosome is flattened and distorted as though subjected to conflicting stresses. Eventually such an X-chromosome will pass to one pole or the other without division, due apparently to the retraction of some of the processes. A count of 115 first spermatocyte metaphases gives the X-chromosome telomitic in 40, atelomitic in 50 and showing evidence of a conflict in 25.

Perhaps the reader should be reminded that in the grasshoppers each egg which produces a male has only one sex-chromosome at the time of fertilization and that is contributed by the mother. All of these variations, therefore, have arisen in this individual. A change from telomitic to atelomitic or vice versa might be due to inversion of a segment of the chromosome. This, however, would not cause a change in the number of fibers. Conceivably, the extra fibers might be acquired by translocations involving spindle fiber loci from other members of the complex but in that case a cell in which the X-chromosome has fibers arising from three points should show two of the other chromosomes behaving eccentrically because of the loss. Such abnormalities are not present.

Naturally a study of these multiple spindle fibers and of shifts in position of the fibers lead to a consideration of the origin and nature of the fibers which pass from the chromosomes to the poles.

In 1917 I reported (p. 459) and figured (Plate 7, Fig. 45) a single X-chromosome in *T. fallax* which showed several distinct fibers passing towards both poles of the spindle. At that time, I simply recorded it as an unmistakable fact with no surmises as to the cause. It is reproduced as Fig. 2 of the present paper for the convenience of the reader and for comparison with other X-chromosomes which exhibit a similar phenomenon.

Figure 3 is from the same individual as Fig. 1. Note that the apex of the V is marked by a group of delicate fibers. Schrader has emphasized the existence of a like condition in other forms. Figure 4 is from *Derotmema laticinctum,* a species which usually has no atelomitics. Here, I think, the solution of the problem becomes self-evident when we compare this X-chromosome with the large tetrads in diakinesis just below it (Figs. 6 and 7). Delicate pseudopodial-like processes which contact either those from other chromosomes (Fig. 7, *A, B*) or the nuclear membrane where they form enlarged or even plate-like

contacts (Fig. 8) are put forth at this stage,[4] apparently, from every chromomere except those which resemble the X-chromosome in precocity. These processes can be retracted almost instantly as is attested by the chromosomes of contiguous cells which show transitions such as are illustrated in Figs. 6, 7 and 11. This cyst was fixed, fortunately, at the moment when the cells were passing from late diakinesis to early metaphase; in a few cells the nuclear membrane had disappeared and chromosomes, only slightly smoother than the tetrad at the right in Fig. 11, were forming the equatorial plate. Short processes comparable to those on the X-chromosomes shown in Fig. 9 are evident, occasionally, on such tetrads at the spindle fiber loci. The whole testis appears to be normal so that I believe the variations shown to be the regular occurrence. In fact, tetrads of like density, as denoted by staining reaction, vary within the same nucleus in regard to the degree of contraction or extension of their processes and even the parts of a single tetrad exhibit this diversity, as may be seen in Fig. 6, where the ring-shaped tetrad has some processes long and attenuated and others short and blunt as though they had been retracted suddenly. Figure *7A* is an end view of one arm of a tetrad which illustrates the fact that the processes are sent out not only at right angles to the plane of the next division but in various directions. Club-shaped ends on processes which have severed their contacts, such as are illustrated at *7B*, suggest that external stimuli applied to the process itself may cause retraction as well as intrinsic forces which presumably usually cause their withdrawal.

Figure 9 shows two late diakinesis X-chromosomes in their actual position relative to each other. Note that they are as fully condensed as the tetrads are at metaphase. The single process on each is in the usual position for the spindle fiber of the X-chromosome in this species and in these instances is certainly of chromosomal origin. Figure 10 from the same cyst represents an X-chromosome which has maintained an end-to-end association with a tetrad up to this late stage, and I suspect such an occurrence accounts for the telomitic X-chromosomes in this species. Figure 12 is from an earlier cyst. Approximately half of the X-chromosomes are U-shaped and half rod-shaped in this stage. At first thought one might assume that such forms are the direct precursors of the V and rod-shaped X-chromosomes, respectively, of the metaphase, but this is improbable because in all species of short-horned grasshopper which I have observed, cytologically, the X-chromosomes

[4] To obtain these processes at their best a number of precautions are necessary. First the animal should be killed in a suitable manner such as the application of xylol to the spiracles, next the testes should be removed from the body with delicacy and care in order to avoid unnecessary shock and quickly transferred to an abundance of good fixative. Finally the stain must not be extracted too much, the processes are chromophilic but very attenuated.

form close U's at about this stage and a little later thick rods, regardless of whether the X-chromosomes at metaphase are telomitic or atelomitic.

With the sex-chromosomes at their maximum density during late diakinesis and beginning to become diffuse just before the metaphase and with the roughened diakinesis tetrads becoming more compact as they approach the metaphase, a brief period occurs when the two types of chromosomes present similarly roughened contours. Such a stage is shown in Figs. 13 (X-chromosome below), 14 (X-chromosome above), 15 (X-chromosome only) and 11 (tetrads). Compare also the tetrad in Fig. 14, on which corresponding processes are still evident on sister chromomeres, with the sex-chromosome from *Circotettix verruculatus* shown in Fig. 5 which has put forth a process toward both poles for each of five aggregates of chromomeres.

The foregoing observations seem sufficient to raise a strong presumption that the chromosomal fiber of the sex-chromosome is a process put forth by the chromosome itself.[5]

Let us now consider some later stages; first, the metaphase spindle, next conditions which immediately precede the metaphase, and finally the anaphase. Naturally, many of the points which I shall mention have been observed by other cytologists. Four critical features, however, seem to have escaped notice. Attention will be called to them in the following pages.

In lateral views of well-formed metaphase spindles, two kinds of fibers are evident on a morphological basis. The continuous fibers, which in the grasshoppers do not form a central spindle but are intermingled with the other spindle components, are slender, of uniform thickness and, in fixed material, somewhat wavy. The chromosomal fibers run a straighter course and are much heavier at their loci of emergence from the chromosomes, from which they taper gradually towards the centrioles,—thus, they vary in thickness throughout their length. These differences are illustrated in Fig. 18 from a spermatogonium of *Circotettix rabula* and Figs. 24 and 25 from first spermatocytes of *Trimerotropis citrina*. Convinced that the chromosomal processes pos-

[5] At this place I should like to call attention to another point. The first three drawings in Fig. 11 are of the same tetrad in the size series from three cells. In addition to showing the transition of contour already mentioned, they are of interest on account of the characteristic spherules which mark this tetrad in late diakinesis and which probably are either secretory or excretory products. They are usually embedded in the surface chromatin but may be at a considerable distance from the parent chromosome with which they are still connected by what appear to be delicate fibrils. Later these connections may disappear, leaving some of the spherules free in the cytoplasm where for a time they continue to give the Feulgen reaction. I believe, for reasons which I hope to develop in a later paper, that they are metabolic products and not true chromatin in process of elimination.

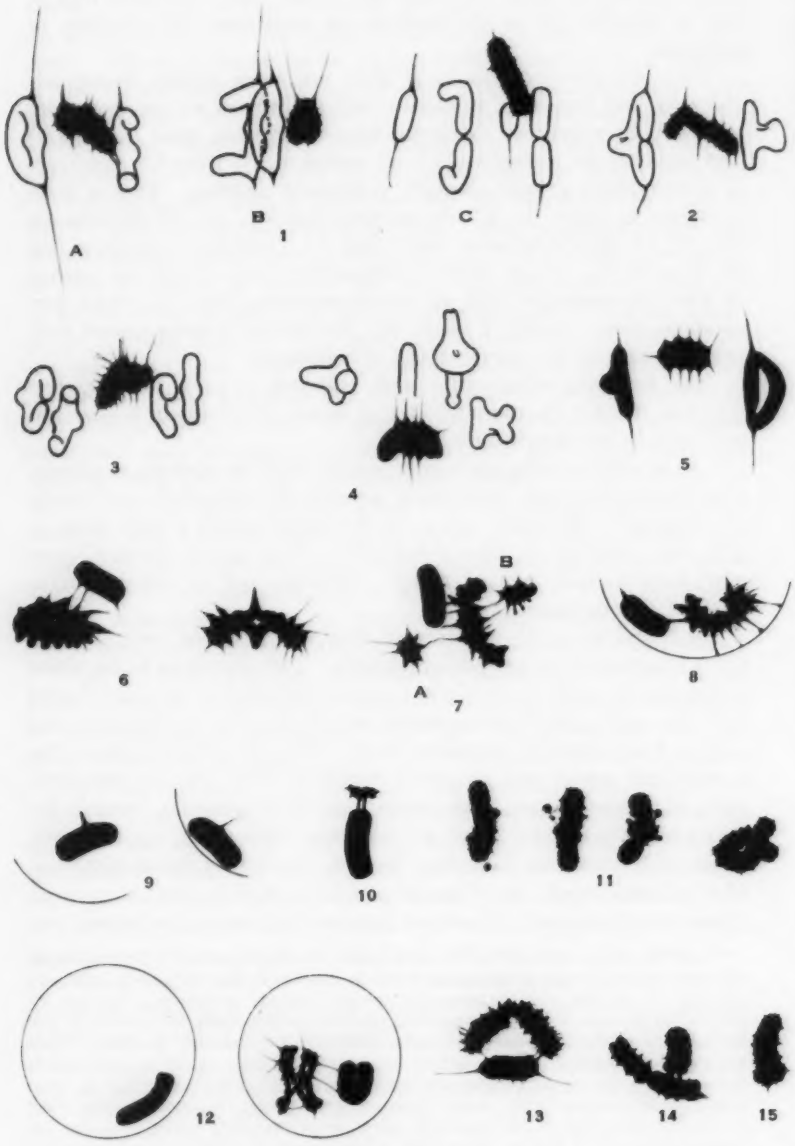

PLATE I

sess considerable elasticity by evidence such as is represented by Figs. 23 and 29, which would be difficult to explain on any other basis, I expected them to become thicker as they became shorter in anaphase. Such, however, is not the case normally. The processes, beginning with the heavier basal parts, apparently, are reincorporated into the anaphase chromosomes as they move towards the poles, so that as they

EXPLANATION OF PLATES

All of the figures were drawn with the aid of a camera lucida at a magnification of 2,800 diameters. They were reduced one-third in reproduction.

Explanation of Plate I

Fig. 1. Partial complexes from three contiguous first spermatocyte metaphases, lateral views. X-chromosome, solid black. At *b* it is atelomitic, at *c* telomitic and at *a* the telomitic process is evident at lower right end which is of normal thickness, the apex of an atelomitic V with two processes is indicated above while the other end is flattened and has processes towards each pole. *Trimerotropis coquilletti.*

Fig. 2. Partial complex of a first spermatocyte lateral view, *T. fallax.* Note the three prominent processes of the X-chromosome.

Fig. 3. Similar to preceding. X-chromosome with numerous fibers or processes. *T. coquilletti.*

Fig. 4. Similar to Fig. 3. *Derotmema laticinctum.* Compare this X-chromosome at metaphase with the diakinesis tetrads shown in Figs. 6, 7, and 8.

Fig. 5. Similar to above, except the tetrads also are in solid black. *Circotettix verruculatus.*

Figs. 6–15. Late first spermatocyte prophases, from *T. coquilletti,* showing transition of X-chromosome from the smooth contour characteristic for it in diakinesis and for the tetrads in metaphase (Figs. 6, 7, 8, and 12) to a roughened contour in very late diakinesis (Figs. 13, 14, 15). Note the long pseudopodial-like processes of the tetrads (Figs. 6, 7, 8) which are withdrawn at the approach of metaphase (Figs. 11, 12 and 13).

Fig. 6. X-chromosome and tetrads. Note blunt processes of the ring at left where the fibers have been retracted.

Fig. 7. Similar to Fig. 6. End views of arms of two tetrads at *a* and *b.* Processes go out in all planes and contact those of other chromosomes. Note the knobbed ends of retracting processes at *b.*

Fig. 8. Similar to above. Processes of tetrad in characteristic contact with nuclear membrane.

Fig. 9. X-chromosomes from two adjacent cells. Note the process on each and compare with spindle (chromosomal) fibers of tetrads shown in Fig. 5.

Fig. 10. X-chromosome from the same cyst which will probably be telomitic at metaphase.

Fig. 11. Tetrads from a cyst where metaphase plates are forming in some cells. The droplets associated with the three examples of one tetrad are characteristic. They may be either secretion or excretion.

Fig. 12. Nuclei from two adjacent cells, earlier diakinesis. X-chromosomes at this stage are either rod or U-shaped in about equal numbers.

Fig. 13. X-chromosome and tetrad approaching each other in roughness of outline. Very late diakinesis.

Fig. 14. The same, a trifle later stage.

Fig. 15. X-chromosome in very late diakinesis. Compare with X-chromosome in metaphase shown Fig. 1, *c.*

shorten they become more delicate. The linear order of their constituents is thus preserved and there is no loss to the chromosome. It seems to follow that the chromatids move apart through some inner mechanism and that the chromosomal processes act more as guides than as "traction fibers."

If we turn now to premetaphase conditions, we find that as early cytologists observed, the nuclear membrane disappears first on the side traversed by the separating centrioles. Some early workers also noticed that the fibers from the two asters intersect in the plane of the equator at early metaphase, but later curve inward and become part of the central spindle. A characteristic which seems to have escaped mention, however, is that in this series of actions one side is still slightly in advance of the other (see Figs. 19 and 24). This condition applies also to the arrangement of the chromosomes on the spindle. The first of the critical features mentioned above is associated with this fact. (1) In lateral views of very early metaphases when the equatorial plate is forming, the chromosomes on one side of the spindle may have typical chromosomal fibers extending from the chromosomes to the poles, while on the other side identical fibers (processes) extend from the chromosomes in the general direction of the poles, but are not yet oriented towards the centrioles. Such a case is illustrated in Fig. 16. The lower process on the small rod at the left is of especial interest because it bends outward towards the cell membrane crossing that of the larger, underlying tetrad. These structures are identical so far as they go with the unquestionable chromosomal fibers on the two tetrads at the right. Their homology with the processes on the sex-chromosomes

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EXPLANATION OF PLATE II

FIG. 16. First spermatocyte metaphase, lateral view. Chromosome fibers reach the poles on one side, forming and not yet oriented on the other.

FIG. 17. Similar to above. Note separation of the processes; also, that they are thicker at point of emergence from chromosomes.

FIG. 18. Spermatogonial metaphase, lateral view, spindle very uniform. Chromosomal fibers thicker and straighter than continuous fibers. *Circotettix rabula.*

FIG. 19. Second spermatocyte, lateral view, from same individual. Processes pass straight out from chromosomes until they approach the poles, then bend abruptly towards them.

FIG. 20. Two tetrads where the point of spindle fiber origin is different for each homologue. *T. fallax.*

FIG. 21. Six tetrads in anaphase illustrating interzonal fibers. *T. fallax.*

FIG. 22. Three tetrads in metaphase showing relation of chromomeres to chromosomal fibers. *T. fallax.*

FIG. 23. Tetrad displaced in sectioning. Chromosomal process distorted but unbroken. *T. fallax.*

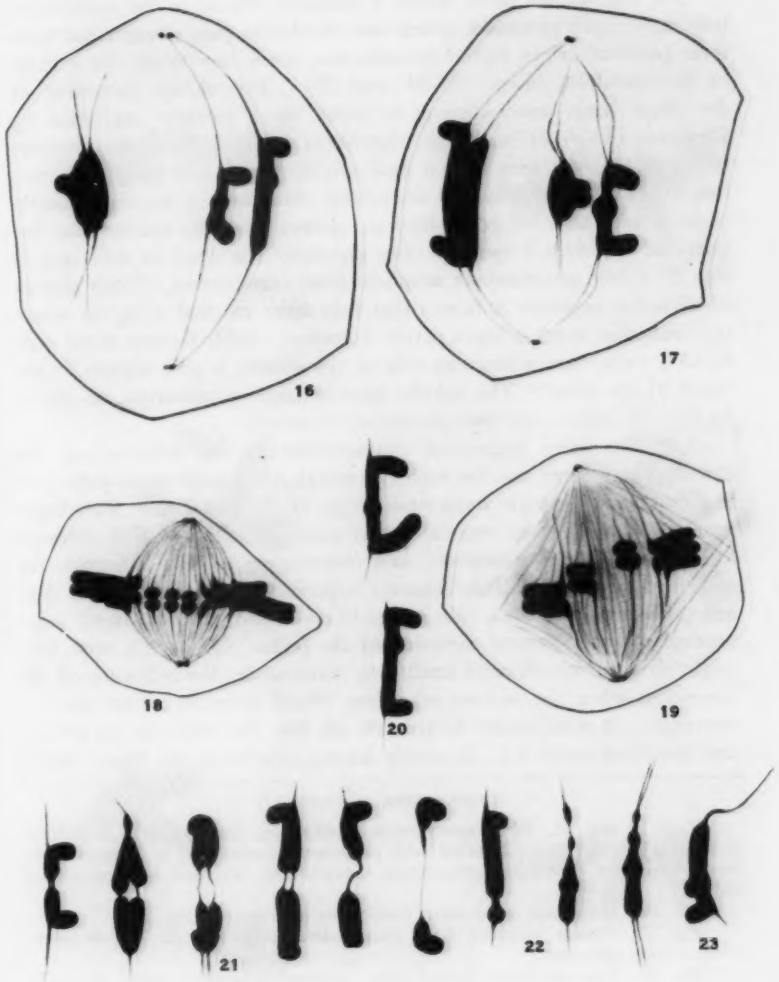

PLATE II

shown on Plate I, especially Fig. 9, and on the tetrad at the right in Fig. 6 seems almost equally certain.

(2) The second point which I consider critical is the sudden inbending of such processes, which have tended to pass straight out from their point of origin in the chromosome, when they reach the vicinity of the centrioles (Figs. 19, 24, and 25). This abrupt inbending of the fibers lends some support to older views recently supported by Cleveland (1934, 1935*a*, 1935*b*) that fibers which lead from the chromosomes to the centrioles have a dual origin. I am convinced, however, that in the grasshoppers they are wholly chromosomal in origin; partly because, even near the poles, they are always markedly heavier than the fibers of the central spindle. See especially the dyad at the right in Fig. 29, a first spermatocyte anaphase from *Dittopternis*. Their change of direction probably indicates that they have reached a region where the centrioles exert a more active influence. Such figures occur only in early metaphase where one side of the spindle is still slightly in advance of the other. The spindle later becomes symmetrical, as shown by Fig. 18 from a spermatogonium of *C. rabula*.

(3) The third suggestive characteristic is the behavior of the chromosomal fibers, one for each chromatid, often evident on each dyad in the first spermatocyte metaphase (Figs. 17, 21, and 23).[6] They taper gradually towards the centrioles and may diverge for some distance after leaving the chromosome, later converging as they approach the centrioles (Fig. 17). This behavior is perfectly comprehensible if they are processes put forth, one by each chromatid, which extend independently, in the general direction of the poles. On such a view they might diverge considerably until they come under the influence of the centrioles, when the sudden inbending would naturally cause them to converge. It need hardly be pointed out that this behavior, as well as that described under "2," is wholly incompatible with any theory which

EXPLANATION OF PLATE III

FIGS. 24 and 25. First spermatocyte metaphases, lateral views, *T. citrina*. Individual represented in 24 killed with potassium cyanide, that in 25 with xylol. Such differences in spindle configuration characteristic for these two methods of killing.

FIG. 26. Metaphase, polar view, from same individual as Fig. 24.

FIG. 27. Similar to above, from same individual as Fig. 25. Chromosomes form a more open plate.

FIG. 28. Late anaphase, lateral view, chromosomes surrounding the poles. Same individual as Figs. 24 and 26. Spindle .69 of the length attained at metaphase when animals are killed with xylol.

FIG. 29. One pole of an anaphase. One chromosome and its fiber in plane of section. *Dittopternis turbata*.

[6] Henking (1890) noted this condition in first spermatocytes of *Pyrrhocoris* and recognized it to be associated with the valence of the chromosomes.

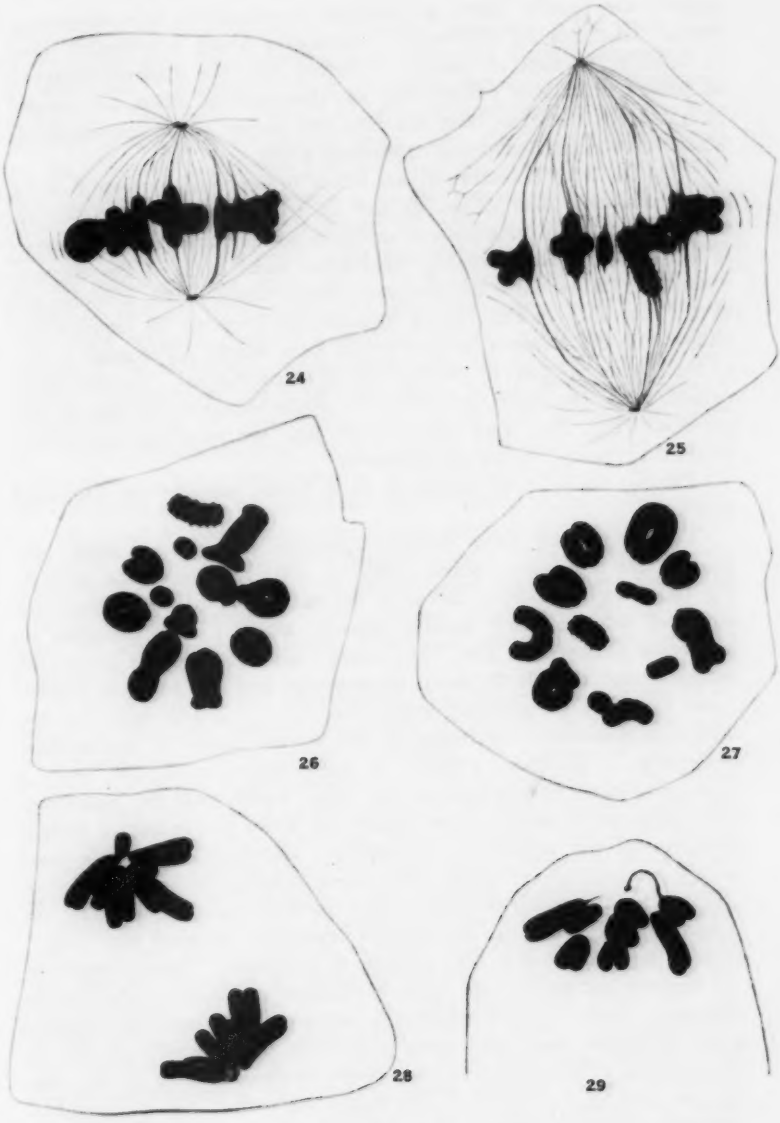

PLATE III

regards the spindle fibers as artifacts, produced in a homogeneous spindle substance by differential coagulation along hypothetical lines of force at the time of fixation. Neither does the observed behavior fit Bělař's hypothesis (1929) that the chromosomal fibers are formed by a mucous secretion from the insertion points of the chromosomes which flows along underlying continuous fibers.

(4) The fourth feature is the actual presence of chromatin along the chromosome fiber (Figs. 22 and 23, tetrad at the left). The resemblance of these structures to satellites and constricted chromosomes is self-evident. Furthermore, the thicker basal parts of the chromosomal fibers often show the chromatic stain (hæmatoxylin, safranin and even Feulgen). In Fig. 16, for instance, the fibers on the tetrad at the right retain the safranin for nearly half their length. The same is true of the heavier portions of the fibers in all of the figures. As was stated earlier, all of the slides used with the single exception of one of *Dittopternis* (Fig. 29), were stained more than ten years ago and differentiated primarily for the chromosomes.

Passing finally to the anaphase, let us briefly consider the interzonal fibers.

Two or possibly three distinct structures are confused under this term in the literature. Mark (1881, p. 198) introduced the term in the following sentence: "Between the two zones of thickenings are stretched delicate nearly parallel threads, which I shall designate as *interzonal filaments.*" The "two zones" are the anaphase groups of chromosomes, though the word chromosome had not yet been coined. Later (p. 230) he definitely states that the ". . . free ends (of the interzonal filaments) terminate in the lateral zones of spindle-fiber thickenings." Hermann (1891), on the contrary, concluded, from a study of mitosis in salamanders and other forms, that the connecting fibers apparent in anaphase are simply the fibers of the central spindle revealed by the separating chromatids. Hermann's view has been widely accepted by cytologists who failed to recognize the fact that in addition to, and morphologically quite distinct from, the fibers of the central spindle, there are connections frequently between the "free" ends of the chromatids as they separate in anaphase. These connections are thickest just as the ends begin to separate and at this time take the chromatin stain. As the separation increases the connection becomes more attenuated and, consequently, destains more rapidly so that in preparations differentiated for metaphase chromosomes all of the stain may be removed. A series of such stages is shown in Fig. 21 from first spermatocytes of *T. fallax*. The sixth tetrad from the left shows a connection which has persisted longer than usual. This tetrad is of further

interest on account of the way in which the chromatin of the upper dyad is drawn out along the connecting fiber, a behavior directly comparable to the extension of chromatin along the chromosomal processes illustrated in Fig. 22. Ordinarily, in the Acrididæ, the interzonal connections separate (I do not believe that they break) near the mid-region at about the stage shown in the third tetrad from the left and are retracted each end into its own chromatid. To me, the action seems to be exactly what one would expect under the existing conditions; namely, flexible, viscous, protoplasmic rods are separating in such a manner that the final contact is end to end. As these ends are separating, it is readily conceivable that viscous protoplasm belonging to each might be drawn out between them to a varying degree, depending upon such factors as the rapidity of the movement and the viscosity of the chromatids. Whether or not interzonal fibers occur in normal, actively dividing cells may be questionable, but they certainly do occur in living cells which are sufficiently normal to complete division as well as in fixed preparations. If my conception is correct, they may well be lacking both in entire groups of organisms and in special cell divisions where the rigidity of the metaphase chromatids is adequate to prevent adhesion, or where the chromatids are separated widely. For instance, in the second maturation division of the short-horned grasshoppers, interzonal fibers are lacking entirely. This condition I believe to be due to the relatively wide separation of the chromatids when they come into the plate for this division.

In my material I find no evidence of a chromosomal sheath or of tubular connections between separating chromatids in anaphase such as Schrader describes in certain Hemiptera. In *Leptocoris* first spermatocyte anaphases fixed in Flemming's strong solution, I have found no interzonal fibers but a pathway such as the dense daughter chromosomes might leave when separating from each other in a fairly viscous cytoplasm if the clearer, more fluid part of the cytoplasm flows in between them as they move apart. This condition is essentially the same as Ellenhorn (1933, p. 300) reported in living cells from *Tradescantia* anthers, where the cytoplasmic viscosity had been increased by pressure sufficient to cause a reversible gelation which did not stop the mitotic process. Schrader (1934, p. 520) states that Ellenhorn, like himself, interprets the interzonal connections as tubes. Fundamentally, their conceptions are entirely different since Ellenhorn considers the internal connections to be transient canals through viscous cytoplasm and Schrader (1932, p. 537) believes them to be a chromophobic outer layer of the chromosome which is pulled out in the form of a tube between the separating daughter chromosomes and which may persist

with the lumen obliterated until the formation of the spindle for the succeeding division.

A further phenomenon which must be concerned with the structure of the spindle, although I do not quite see its mechanism, should be considered here and that is the variation in size of the spindle which may be produced experimentally. Figures 24 to 28 are from first spermatocytes of *T. citrina*. Figures 24, 26, and 28 are from the same insect which was killed with cyanide fumes. Figures 25 and 27 are from another individual which was killed with xylol applied to the spiracles. The differences illustrated are characteristic. Cyanide gives short spindles with the centrioles well removed from the cell membrane and much of the cytoplasm apparently not involved, while xylol gives approximately twice as much distance between the centrioles which are consequently near the cell membrane with the spindle occupying nearly the entire cell. Correspondingly, the chromosomes are more crowded together in a smaller equatorial plate in the former case, as may be seen by comparing Figs. 26 and 27 from the same two insects. Even at the completion of the anaphase the distance between the poles in cyanide-killed animals is only slightly more than two-thirds of what it is in metaphase for an animal killed with xylol (Fig. 28).[7] Some question has existed as to which is the normal condition but recently an individual of *Dittopternis* killed with xylol gave both types of spindles. The short ones occur in a limited region which was injured, evidently, when the testis was removed. The cells have coarsely granular, muddy staining cytoplasm and in extreme cases the chromosomes had fused into a pycnotic mass characteristic of dying cells. Clearly, then the large spindles are the normal ones.

When one considers that less than five minutes previous to fixation both animals, from which the figures were taken, were presumably in the same condition so far as their mitotic spindles were concerned,[8] one marvels at a mechanism which permits such rapid rearrangement. If the chromosomal fibers are processes from the chromosomes, their rapid retraction is understandable. As to the astral rays and continuous spindle fibers, one can imagine elongate molecules to be involved. A. R. Moore (1935) has produced evidence for the existence of such elongate prestructural elements, molecules or micelles, in protoplasm by showing that the plasmodia of *Physarum* will pass through moist walls of hard filter paper with pores about 1 micron in diameter uninjured while they

[7] McClung (1918*a*) reported the relation of this condition to the method of killing the animal but illustrations were not given.

[8] This presumption is based on some fifty specimens killed with cyanide fumes and thousands with xylol.

are killed if forced through silk gauze with pores less than 200 micra in diameter.

Discussion

The observations of the earliest investigators in any given field frequently are strictly accurate and amazingly detailed. Probably because they alone approach the subject with a completely open mind.

It is interesting to note that Flemming (1882, Pl. VI, Fig. 3*b*) shows a late anaphase of a living "Leydischen schleimzelle" of a salamander larva with achromatic threads, which he states are clearly visible. The general accuracy of Flemming's observations is attested by his figures of mitosis from fixed material which far excel those of many later cytologists. Certainly, then, his statement that he saw achromatic threads, which his figure shows to be in the interzonal region, is to be taken at face value. Nevertheless, they might have been any one of three structures: (1) continuous spindle fibers, although this is not probable, (2) interzonal fibers which as drawn out parts of the chromosomes should be visible in living cells, since the chromosomes themselves are visible; (interzonal fibers, however, seldom persist until late anaphase); (3) mitochondria which are present in abundance at this stage in most forms and are readily visible in living cells.

Bělăr (1929, Pl. I, Figs. 14 and 15) shows photomicrographs of what is probably a similar condition in living first spermatocyte anaphases of *Chorthippus lineatus*. He interprets the apparent fibers as streaming mitochondria. On the other hand, the excellent ultraviolet photomicrographs of a similar stage in second spermatocytes of another grasshopper, *Melanoplus femur-rubrum*, by Lucas and Stark (1931), show nothing comparable to fibers in the interzonal region. These photomicrographs have been cited by some investigators as evidence that the mitotic spindle is homogeneous. In reality, they are evidences only that any structures which are present are completely permeable to ultraviolet rays in this species. Mitochondria almost certainly were present. In addition, the only anaphase figures are of second spermatocytes (Plate III, Figs. 27, 28) where, as I have mentioned previously, interzonal fibers are lacking. Incidentally, it should be noted that the refractive index of living chromosomes varies greatly in different species of Acrididæ. Consequently, some are more favorable than others for the study of mitosis in living cells.

Knowledge of the various spindle components has been augmented greatly by the work of Cleveland and his collaborators on the flagellate parasites of the wood roaches. There is essential agreement in our conclusions. The chief point of difference is in regard to the chromo-

somal fibers which Cleveland et al. believe to be of dual origin; the intranuclear part derived from the chromosomes and the extranuclear portion from the astral rays. In the grasshoppers, I am convinced that they are entirely chromosomal in origin. While such a divergence would not be surprising in organisms so widely separated, phylogenetically, as the flagellate Protozoa and the grasshoppers, the possibility that the extranuclear chromosomal fibers in these flagellates are put forth from the "chromatin knobs" on the nuclear membrane would explain some of the difficulties encountered on the assumption of Cleveland and his coworkers. For example, describing the anaphase movement of the chromosomes, they state (p. 234) ". . . the extranuclear chromosomal fibers are greatly shortened, while the intranuclear chromosomal fibers are not greatly altered (Figs. 53, 56–58). The shortening of the extranuclear chromosomal fibers is difficult to understand. If, as all other observations indicate, they are astral rays that have been converted into chromosomal fibers by becoming fastened to the knobs on the nuclear membrane, why do they shorten while the unattached astral rays do not?" If the knobs send processes (fibers) through the nuclear membrane to the centrioles such fibers would not be expected to behave as do the astral rays, since their origin would be from the chromosomes. The paragraph just quoted continues: "The unattached astral rays, as a rule, are considerably longer than the longest attached ones (extranuclear chromosomal fibers). This is due to the increased length of the astral rays after the intra- and extranuclear chromosomal fibers are united and will account for the fact that the extranuclear fibers sometimes appear slightly larger than the astral rays, if the chromosomal fibers increase in thickness as the astral rays increase in length." This explanation of the fact that the chromosomal fibers are thicker than the astral rays seems rather far-fetched. If the latter originate from the centrioles and the former from the chromosomes, correspondence in thickness would not be expected.

As is well known, certain workers believe all spindle fibers to be artifacts. Schrader (1932, 1934) reviews the work of a number of such investigators in a very fair and sympathetic manner and gives an adequate bibliography. Schrader's answer to such views is a series of experiments on the mitotic figures of higher forms which show that the components of the half spindle may be bent, independently of each other, by centrifuging and that such metaphases will complete their division; thus disproving, so far as the chromosomal fibers are concerned, the contention that the fibers seen in fixed preparations are artifacts caused by the differential coagulation along lines of force of a homogeneous spindle substance.

If one grants for the sake of argument that lines of force could cause such coagulation artifacts to result from fixation, one would at least expect them to be continuous from pole to pole and of uniform diameter, whereas the outstanding characteristics of chromosomal fibers are that they taper from their points of emergence from the chromosomes towards the poles, and that they are not continuous from pole to pole. In short, a great many of the arguments put forward by those who question the actual existence of spindle components of all categories would never have been advanced if their proponents had had adequate training in the study of mitosis in well-fixed material before undertaking the more difficult study of mitosis in living cells.

Perhaps a brief statement of my conception of a chromosome is in order. In the first place it is an individualized mass of living substance (protoplasm). We speak of the protoplasm of an amœba as being specialized into ecto- and endoplasm and the evidence indicates that the protoplasm of a chromosome is specialized in much the same way. The cortical part must meet its environment adequately. During metaphase, anaphase and early telophase the immediate environment is the cytoplasm and the condensed chromosome exposes the least possible surface. These stages are concerned primarily with increase in cell number and not with the welfare of the chromosomes as individuals. In late telophase the chromosomes form the nuclear membrane (Wenrich, 1916, pp. 86–88). That the chromosomes are the determining factors in this process is further indicated by the fact that in the spermatogonial telophases the sex chromosome of the short-horned grasshopper forms an almost separate and relatively much more commodious "vesicle" for itself while the euchromosomes are rebuilding the nuclear membrane.[9] From the late telophase through the "resting period" and up to the end of the prophase the immediate environment of the chromosomes is the nucleoplasm. In this sheltered situation they expose a greatly increased amount of surface and probably reach the apex of their functional activity and individual well-being. Even this protected environment changes as tissue differentiation progresses and this may well be one of the factors which limit the number of cell divisions, and consequently, the size of a given organ. Support for such a view is found in the fact that in mature tissue cells which resume mitotic activity to repair an injury, the cytoplasm first dedifferentiates. In brief, a chromosome is a living entity clearly capable of growth, of reproducing itself by longitudinal fission, and of amœboid movement.

Finally we come to the crucial point. Can the position of the

[9] McClung (1918*b*) noted the increased diffusion and presumably greater activity of the sex-chromosome during the spermatogonial interphases.

chromosomal fibers change without inversions or translocations of parts of the chromosomes; in other words, can new loci arise and suppress the older ones? If so, what is the mechanism involved? The association of the chromosomal fibers with a fixed region of the chromosome is one of the most constant features of chromosomal organization. Cytogeneticists, however, have long known that experimental breakage and fusions of chromosomes due to irradiation, abnormal temperatures and unknown intrinsic causes may result in shifts in the location of the chromosomal fiber region so that it does not invariably occupy the same position in a given chromosome; but in such cases, the region maintains its characteristics whatever its location.

The first well-founded case of an association of the chromosomal fibers with a different region (chromomere in this instance) is that noted by Wenrich (1916) in *Phrynotettix*. His figure 65h–m illustrates six tetrads of his type C_1. Those shown at h–j are dividing equationally, those at k–m reductionally. In the former case the ends marked by the polar granules (Pinney, 1908), as shown by a comparison with the earlier stages, are as usual oriented towards the poles. In the latter case the large distal granule, characteristic of the free end of the larger homologue in the spermatogonial telophases, is oriented towards one pole and the erstwhile free end of its homologue is, of necessity, directed towards the other pole. In other words, the chromosomal fibers have shifted from the ends of these homologues marked by the polar granules to what in the spermatogonia were the distal or free ends, one of which is marked by a pronounced "distal granule." Wenrich found this change to have taken place in approximately fifty per cent of the 928 first spermatocytes recorded in the individual under consideration. His close study of the earlier stages of this tetrad renders it highly improbable that any inversion of parts of a chromosome where the regions are so well-marked could have taken place in half of the cells without detection by so competent an observer.[10]

Wenrich also notes (p. 84) the critical point, that the polar granules, located at the spindle fiber ends of the euchromosomes, and the large distal granules of tetrads "B" and "C," as well as the whole of the sex-chromosome become roughened by the metaphase of the first spermatocyte division (Plate IX, Figs. 99 and 100) and suggests that some

[10] Schrader, F. (1936), in a paper which appeared after this manuscript was sent to press, concludes on the basis of structure and staining reaction that the "kinetochore" is quite distinct from an ordinary chromomere. I have no doubt that the degree of specialization varies in different organisms. However, I cannot agree with his suggestion that the polar granules of *Phrynotettix* (Pinney, 1908, Wenrich, 1916) are not "kinetochores" simply because tetrad "B" possesses a distal as well as a polar granule. In my opinion this is exactly the mechanism by which tetrad "C" shifts its spindle fiber locus.

common physical or chemical properties underlie this correspondence in behavior.

One physical characteristic marks all of the above structures as well as sex-chromosomes, chromoplasts and precocious chromomeres in general; their rhythm of condensation and diffusion is different from that of the rest of the chromatin, with the result that at the metaphase they are already beginning to become diffuse. The X-chromosome of *Stauroderus scaleris* has reached a vesicular telophase condition at the first spermatocyte metaphase (Corey, 1933). Likewise, the X-chromosome of *Notonecta indica* goes into the first spermatocyte metaphase in a diffused condition and with multiple spindle fibers as was shown by Browne (1916, Pl. 6, Fig. 95). Carlson (1936, p. 132) gives a good description of the process of diffusion of the "megameric" chromosomes in the cryptosome stage in seven related genera of short-horned grasshoppers. If precocity of definite chromomeres is associated with the ability to begin putting forth processes at the metaphase, a change (mutation) in the rhythm of activity of certain chromomeres might result in some which are usually relatively quiescent at the metaphase, speeding up and sending out processes capable of becoming chromosomal fibers with a resultant struggle for supremacy such as is exhibited by the X-chromosomes with multiple chromosomal fibers illustrated in Figs. 1 to 5. In my opinion, a specialized chromomere of each chromatid normally sends out a fine pseudopodial-like process at the metaphase which becomes the chromosomal fiber, but a change of rate of condensation and diffusion may result in a shifting of this function to a more precocious chromomere.

Summary

The evidence presented is believed to justify the following conclusions: (1) Chromosomal fibers (half-spindle fibers) are in reality pseudopodial-like processes. They are put forth by definite chromomeres, therefore normally are fixed in position. (2) Due to mutation, using this term in a broad sense, some other chromomere may assume this function with a resultant change in position of the spindle processes which does not involve either inversion or translocation of chromosomal segments. (3) At anaphase the processes, beginning with the heavier basal parts, are reincorporated into the sister chromatids as they move towards the poles. (4) The interzonal fibers behave as one would expect under the existing conditions; namely, flexible, viscous protoplasmic rods are separating in such a manner that the final contact is end to end. As these ends separate the viscous protoplasm belonging

to each is drawn out between them in varying degree depending on such factors as the rapidity of the movement and the density of the chromatid. (5) Both chromosomal fibers and interzonal fibers are integral parts of the chromosomes and return to them during anaphase without loss of chromatin or linear derangement of their constituents. The work is based on six species belonging to the following four genera of Acrididæ; *Trimerotropis, Circotettix, Derotmema* and *Brachystola.* In addition, the paper contains a summary of what are believed to be well-established facts concerning the various components of the mitotic spindle.

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SOME FRESH WATER PROTOZOA WITH BLUE CHROMATOPHORES

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Among the Protista blue is one of the rarest of the colors. The blue-green algæ may be grass-green or shades of brown or red. A culture of a marine *Spirulina* I maintained for two years was constantly violet. Such variations in the color of the Myxophyceæ are due to combinations of chlorophyll, phycocyanin, and phycoerythrin, and to the make-up of the gelatinous envelope at times.

Some Protozoa have a blue or violet color diffused through the cytoplasm as the ciliate, *Stentor cæreuleus* Ehrenberg. Other Protozoa may occasionally have a blue color due to specific pigments contained in chromatophores. A single fresh-water rhizopod, *Paulinella chromatophora* Lauterborn, fulfills this condition, but most of the blue Protozoa are Mastigophora. Various marine Dinoflagellida have blue or blue-green plastids, but according to Eddy (1930) none of the fresh-water armored species in the United States are so colored and I have been unable to find a record of such a species of *Gymnodinium,* the unarmored genus. The genera *Chroomonas* and *Cyanomonas* of the Cryptomonadida have blue chromatophores but Smith (1933) does not record the occurrence of either in this country; Pascher (1913), however, holds *Cryptoglena americana* Davis to be identical with *Cyanomonas americana* Oltmanns. West and Fritsch (1927) record *Chroomonas nordstetii* Hansgirg as reported once from England. From such scanty records of their occurrence it would be imagined that the blue Protista are very rare.

In the past several years I have collected some hundreds of water samples from various field locations. Many of these have been centrifuged to concentrate their microörganisms and examined before great temperature or other changes could occur. In such a sample taken from Newtown Creek at Collingswood, New Jersey, in 1932, I found a blue flagellate in abundance. Since then I have found *Paulinella chromatophora,* a new species of *Chroomonas,* and three other new flagellates with blue chromatophores. I have also noted a rather widespread field occurrence of some of these organisms. Table I shows those which have occurred at nine locations.

From this table a comparative idea of the rareness of occurrence of these blue Protozoa may be obtained. It should be stated, however, that *Cyanomonas americana* has never been seen by me from but two stations, and the same is true of *Cyanomastix morgani*. Species of *Chroomonas*, however, are relatively common; thus bi-weekly samples from a small pool in South Mountain Reservation, Essex County, New Jersey, rarely failed to show some of these flagellates, while in 76 plankton samples from the Muscle Shoals area in Tennessee, Mississippi and Alabama, 21 contained one or more species and in a few they constituted the most abundant protozoan organism. This is a much more frequent occurrence than available records indicate. Their small size

Protozoa with Blue Chromatophores Occurring at Nine Sampling Stations

Name of organism	Locality where found								
	Reservation Pool, Millburn, N. J.	Raritan River, N. J.	Pond, Woods Hole, Mass.	Reelfoot Lake, Tenn.	Reservoir, Wilson Dam, Ala.	Lake Wilson, Ala.	White's Lake, Pickwick Dam, Tenn.	Collier Slough, Florence, Ala.	Yellow Creek, Miss.
Paulinella chromatophora								×	×
Chroomonas pulex	×	×	×	×	×				×
Chroomonas nordstetii	×					×		×	
Chroomonas cyaneus	×				×	×	×	×	×
Cyanomonas americana									×
Cyanomonas cæreuleus							×		
Cyanomastix morgani									×
Gymnodinium limneticum				×		×			

and activity prevents ready identification, unless they are concentrated in some manner.

In all these Protozoa the color is a bright blue, localized in chromatophores from which it diffuses out as a blue liquid after death of the organism. No experimental work on its nature has been done, but it is brighter than extracted phycocyanin.

Paulinella chromatophora Lauterborn. Plate I, Fig. 1.

This rhizopod was found at three stations in the spring of 1936, two of them shown in Table I. Its recurrence was several times noted at one of these. All the stations were clear streams with considerable debris and an abundance of filamentous blue-green algæ. Hydrogen ion concentrations were 6.2, 6.8, and 7.4. The animals conformed rather closely to the published descriptions, but are more pyriform in shape

and with nine rows of plates instead of eleven or twelve. The cell does not fill the test and the cytoplasm is clear and homogenous. The nucleus is visible in the upper portion. All animals had two contractile vacuoles, definite in location as shown. The few pseudopodia are long and branch a few times. They are moved sluggishly. The two chromatophores are curved bands, decidedly blue with no pyrenoids. It is unlikely that they are symbiotic algæ as Kudo (1931) suggests, for every individual contained two; eighteen in one sample alone were examined.

Cyanomastix morgani, gen. nov., spec. nov. Plate I, Figs. 2, 3.

This organism occurred rather abundantly in a fresh water lime sink pond in Morgan County, Alabama, during September, 1935. It is difficult to classify, for its color places it in the Phytomastigoda, and its thick membrane, lack of a gullet and simple vacuole system should place it in the order Phytomonadida. While the peculiar shape and color of the chromatophores, and lack of a stigma are exceptional to the Chlamydomonadidæ, I believe it should be placed at least provisionally in that family. No resting stages or reproductive phases were observed.

Organisms practically uniform in size, 15 to 20 microns in length, 8 to 15 in width, slightly pyriform in shape. Membrane thick with five shallow grooves at round end, a small opening at top. Cytoplasm homogenous with an anterior nipple from which emerge two slightly subequal flagella, the longer about cell length. A contractile vacuole is just below this nipple. No nucleus was observed. All individuals had two chromatophores, band-shaped, twisted or turned, a vivid blue green. On them are a few large bodies resembling pyrenoids with irregular edges. The only noticeable cell inclusions are small spheres, probably oil.

Locomotion is rapid, rather like that of *Chlamydomonas.* The organisms also have the trick of attaching themselves by the anterior nipple, with the flagella opposed. Nutrition seemed to be holophytic.

Chroomonas setoniensis, spec. nov. Plate I, Figs. 4, 5.

This species conforms to the generic description as given by Pascher (1913). In size it is the largest member of its genus, attaining a length of 20 microns. Its flattened oval form, deeply insunk gullet and size seem to merit calling it a new species. It has been common at two stations in the Muscle Shoals area, and in the pool in South Mountain Reservation referred to above, it appeared constantly during several months in considerable numbers.

PLATE I

Cell constant in shape, flattened, oval, slightly obliquely truncate at the anterior end, with a deeply insunk gullet or mouth depression, open along one side. About 20 microns long, 15 wide and 8 thick. Two subequal flagella emerge from the mouth depression, the longest about 25 microns long, the shorter about 20. The single chromatophore is bright blue, having the form of a peripheral cylinder, split from anterior to posterior end, with irregular edges. One simple contractile vacuole near the point of emergence of the flagella. The nucleus is median with a thin membrane and no peripheral chromatin granules. Stained preparations show the flagella to end in small basal bodies, with no visible rhizoplast. There are several types of inclusions, spheres which might be oil, and bodies resembling paramylum. It swims rapidly revolving on its main axis. No evidence of reproduction has been seen.

Cyanomonas cæreulus, spec. nov. Plate I, Figs. 6, 7.

This organism has been seen in only two plankton samples; one from a lake, small and partly shaded, in Morgan County, Alabama, the other from a lake in Hardin County, Tennessee, at Pickwick Dam. This water was densely shaded by cypress and tupelo trees.

Cell somewhat egg-shaped, but flattened on one side. The anterior end is pointed. There is a small depression anteriorly, on the right side; two flagella emerge here. The longer is carried backward, about 20 to 25 microns long, and does not trail on the substratum at all times. The anterior flagellum is about cell length. The animal varies somewhat in size, 15 to 20 microns long and about half as wide. It is somewhat metabolic in that it assumes a discoid shape at times, as when changing direction of movement. The pellicle is not evident and its surface is smooth. The nucleus is small and central; there are no visible cell inclusions but the cytoplasm is finely homogeneously granular. No contractile vacuole is to be found. There are from 15 to 25 bright blue discoid chromatophores, without pyrenoids, small and peripherally located. Its nutrition and reproduction were not noted.

Davis (1894) described a blue-green motile cell from the salt marshes of the Charles River in Massachusetts as *Cyanomonas americana*. He thought it to be a motile cell of the blue-green alga *Polycystis pallida*. In general this organism is like his, except that the depression from which the flagella emerge is lateral instead of anterior, and there are no eyespots. A few flagellates more like his were found in a pool in Tishomingo County, Mississippi, in the summer of 1935, but could be studied only superficially. They came from a small pool which did not seem to have any blue-green algæ in it, and I have found nothing to connect these blue-green or blue flagellates with such algæ. On the contrary, they exhibit a high degree of organization.

Gymnodinium limneticum, spec. nov. Plate I, Figs. 8, 9.

This was the most common dinoflagellate at one station on Reelfoot Lake, Tennessee, in September, 1935. It was eaten in considerable numbers by the larvæ of malarial mosquitoes there.

Cell 25 to 35 microns long, somewhat dorsiventrally flattened, but a regular oval in outline. Hypocone somewhat larger than the epicone. Transverse furrow wide, deep, with no displacement. The longitudinal furrow does not enter the epicone. Trailing flagellum about one and a half times the body length. Commonly a large pusule in the hypocone, the nucleus being subcentral. The blue color is due to 8 to 12 oval discoid chromatophores scattered through the cell. No oil or other inclusions were noted, and as no food bodies were found it is inferred that nutrition is holophytic. The animal is naked and the surface is smooth. No reproduction was seen.

These blue flagellates present a perplexing question as to the significance of their color and its development. It appears improbable that they have any close relationship to the blue-green algæ; they are too highly organized. Morphologically their relationship to amœboid and flagellate Protozoa is unmistakable. Since all appear to nourish themselves holophytically, it is perhaps simplest to regard them as species in which chromatophores have developed containing a blue pigment in place of, or in addition to, chlorophyll.

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THE MOLECULAR WEIGHTS AND PH-STABILITY REGIONS OF THE HEMOCYANINS

INGA-BRITTA ERIKSSON-QUENSEL AND THE SVEDBERG

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Previous ultracentrifugal investigations carried out in this laboratory have brought to light a number of different molecular weights and sedimentation constants among the hemocyanins (Svedberg and Chirnoaga, 1928; Svedberg and Heyroth, 1929, *a*, *b;* Svedberg and Eriksson, 1932; Svedberg, 1933; Svedberg and Hedenius, 1933; Svedberg and Hedenius, 1934). It has also been observed that a change in the pH of the solution often causes the appearance of one or several new molecular species and the disappearance—partial or complete—of the species present before the change was brought about (Svedberg and Hedenius, 1934; Svedberg, 1934, *a*, *b*, *c*, *d*). The pH-stability regions characterized in this way have often been found to be quite different even in the case of hemocyanins possessing the same molecular weight at the isoelectric point. This circumstance seems to be of considerable interest from a biological point of view and we have therefore made an attempt to study in some detail the molecular weights and pH-stability regions of a number of hemocyanins in order to find out whether the diagram giving the molecular weight (or the sedimentation constant) as a function of the pH of the solution could be used to define an animal genus or species.

METHOD

The ultracentrifugal technique developed for the determination of molecular weights and sedimentation constants has been described elsewhere (Svedberg, 1934, *a*, *b*, *c*, *d*). In the case of sedimentation equilibrium measurements the molecular weight is given by the formula:

$$M = \frac{2RT \ln c_2/c_1}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)} \qquad (1)$$

where M = molecular weight, R = gas constant, T = absolute temperature, c_2 and c_1 = concentrations of solute, V = partial specific volume, ρ = density of solvent, x_2 and x_1 = distances to the centre of rotation, ω = angular velocity.

For the determination of c_2/c_1 a procedure based upon measure-

ments of light absorption has previously been used almost exclusively. A new method for determining the concentration gradient utilizing the change in refractive index has been developed by O. Lamm (1933) and has in most cases been found to be superior to the absorption method in its present shape. The refraction method in its two forms (the scale-method and the slit-method) has therefore been used in this investigation.

Sedimentation equilibrium measurements require the expenditure of very much time and work and such determinations can therefore hardly be used for mapping out completely the pH-stability regions. In cases where more than one molecular species is present in the solution the equilibrium method has also the great drawback that it is unable to give definite information about the number and nature of these components. We have therefore used the sedimentation velocity method for collecting the data necessary for tracing the pH-stability pictures supplementing these measurements with equilibrium determinations. In the following diagrams the changes in the sedimentation constant will accordingly indicate the dissociation reactions occurring at different pH values. The sedimentation constant results from a measurement of sedimentation velocity by reducing the directly observed values to standard conditions with regard to centrifugal force, buoyancy and viscosity:

$$s = dx/dt \; 1/\omega^2 x \; \eta/\eta_0 \frac{(1 - V\rho_0)}{(1 - V\rho)} \qquad (2)$$

where dx/dt = observed sedimentation velocity, ω = angular velocity, x = distance from centre of rotation, η = viscosity of solvent, η_0 = viscosity of water at 20° C., V = partial specific volume of solute, ρ = density of solvent, ρ_0 = density of water at 20° C.

In order to minimize the danger of causing artificial changes in the state of aggregation all the hemocyanins have been studied as they occur in the blood without any treatment other than dilution with NaCl or buffer solutions. In the case of the crustaceans the fibrin has been removed by shaking the blood with glass beads. As a rule the light absorption method has been used in the determination of the sedimentation constants (λ = 366 mμ, nickel oxid glass filter or the region λ = 250–290 mμ, chlorine and bromine filter) but some check runs with the refraction method have been carried out. With regard to the details of procedure the previous communication (Svedberg and Hedenius, 1934) should be consulted.

Recent work in this laboratory has shown that some of the earlier sedimentation equilibrium measurements have given too low molecular weight values (probable causes of error: systematic deviations in

the evaluation of the photographic blackenings, incomplete attainment of equilibrium). The discrepancy believed to exist between the frictional constants deduced from sedimentation velocity and sedimentation equilibrium measurements on the one hand and diffusion measurements on the other (Tiselius and Gross, 1934; O. Lamm and A. Polson, 1936) therefore vanishes and the possibility of computing molecular weights by combining sedimentation velocity and diffusion measurements as pointed out long ago by one of us (Svedberg, 1925) and used for such computations at the earlier stages of our investigations (e.g. Svedberg, 1927) is accordingly actualized.

The molecular weight is given by:

$$M = \frac{RTs}{D(1 - V\rho)} \tag{3}$$

where D = diffusion constant and the other symbols have their previous meaning.

A systematic study of the diffusion constants of the proteins undertaken by A. Polson has furnished a number of data also for the hemocyanin. His diffusion constants combined with our sedimentation constants according to equation (3) give molecular weight values which agree very well with those calculated from our sedimentation equilibrium measurements (see Table XXVI).

Sedimentation Constants[1]

The main part of the work consisted in the carrying out of sedimentation velocity measurements at different pH-values. From these data the pH-stability curves were traced. In some cases the reversibility of the dissociation reactions have been tested.

Arthropoda

Hemocyanin is found in the blood of the crustaceans and the Arachnomorpha, but not in that of the myriapods and the insects. We have only studied representatives of the subclasses Malacostraca and Xiphosura, the species belonging to the other subclasses yielding too little blood for an extended investigation.

Crustacea: Malacostraca

The respiratory proteins contained in the blood of the following animals belonging to the order Malacostraca of the class Crustacea

[1] The sedimentation constants are expressed in units of 10^{-13}. Centrifugal force is expressed in terms of the gravitational constant.

The letters A–K are used to characterize the different hemocyanin components. Components having the same sedimentation constant are designated by the same letter.

A + in the column of a table indicates that the component in question has been observed but that it was impossible to calculate its sedimentation constant.

have been investigated: *Pandalus borealis, Palinurus vulgaris, Nephrops norvegicus, Homarus vulgaris, Astacus fluviatilis, Cancer pagurus, Carcinus mænas.* The two sedimentation constants 16 and 23 are characteristic of the group. In *Pandalus* and *Palinurus* the sedimentation constants for the main components are 16.4 and 17.4. In all the others the "normal" sedimentation constant is about 23 and dissocia-

TABLE I

Pandalus borealis

Dilution of blood, 15 times; centrifugal force, 200,000 (speed 52,000 r.p.m.); thickness of column of solution, 0.6 cm.; source of light, mercury arc; light filter, chlorine and bromine; plates, Imperial Process; exposure time, 30 seconds; aperture of lens, F : 36; developer, metol-hydroquinone, 1 minute.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	
HAc, NaAc, NaCl	3.6	0.22		16.1	Containing inhomogeneous components with s_{20} = 12.0 and 4.7
" " "	4.0	0.22		18.4	Containing inhomogeneous components with s_{20} = 4.7
" " "	4.2	0.22	22.3	16.9	
" " "	4.6	0.22	22.4		Inhomogeneous
" " "	5.0	0.22	21.9		Inhomogeneous
" " "	5.2	0.22		17.6	Slightly inhomogeneous
" " "	5.5	0.22		17.4	
KH_2PO_4, Na_2HPO_4, NaCl	6.0	0.22		18.4	
" " "	6.8	0.22		17.4	
" " "	6.8	0.22		17.0	
" " "	6.8	0.22		17.7	
" " "	7.4	0.22		16.4	
" " "	8.0	0.22		16.9	
KH_2PO_4, $Na_2B_4O_7$, NaCl	8.5	0.27		17.0	
" " "	9.0	0.27		17.9	
Na_2CO_3, $Na_2B_4O_7$, NaCl	9.5	0.25		17.7	
" " "	9.5	0.25		17.4	
" " "	10.0	0.25		17.0	
" " "	10.5	0.25		16.6	7 times diluted; NiO-filter
" " "	10.8			18.1	Inhomogeneous lower part
			22.9	17.4	

tion products with sedimentation constants around 16 are formed. *Pandalus* shows an aggregation product with the sedimentation constant 22.9. All the final alkaline-splitting products have sedimentation constants approximating 5.

FIG. 2*a*.

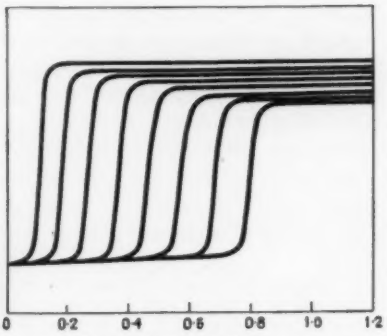


FIG. 2*b*.

FIG. 2. Sedimentation pictures (*a*) with photometric records (*b*) for hemocyanin from *Palinurus vulgaris* at pH 9.0 (s_{20} = 16.4); centrifugal force 120,000; time between exposures 5 minutes.

FIG. 3*a*.

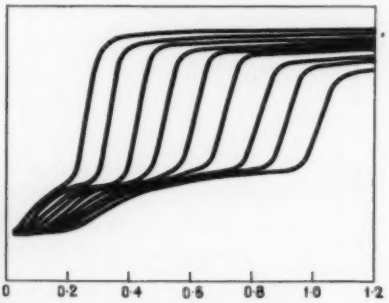


FIG. 3*b*.

FIG. 3. Sedimentation pictures (*a*) with photometric records (*b*) for hemocyanin from *Palinurus vulgaris* at pH 10 (s_{20} = 16.4 and 4.10); centrifugal force 280,000; time between exposures 5 minutes.

Pandalus borealis

Kristineberg, Sweden

The main component of this hemocyanin, component H, has the sedimentation constant 17.4. It is present in the blood from pH 3.6–10.8 with the exception of the pH region 4.2–5.0. It is homogeneous with regard to molecular weight from pH 5.5–10.5. At 10.8, 4.0, and 3.6 a large part of H dissociates into smaller molecules. In the region 4.2–5.0 the blood contains a higher very inhomogeneous

component, G, the sedimentation constant of which is 22.9. Figure 1 (Plate I) gives the pH-stability diagram.

Palinurus vulgaris

Roscoff, France

In the pH-region 3.6–9.4 one molecular species is found in the hemocyanin (component H). This has the sedimentation constant 16.4 and is perfectly homogeneous. At pH 9.4 it dissociates to some

TABLE II

Palinurus vulgaris

Dilution of blood 5 times; centrifugal force, 150,000 (speed 45,000 r.p.m.); in the last two runs 250,000 (speed 59,000 r.p.m.); thickness of column of solution, 2.2–1.0 cm.; light filter, nickel oxid glass; time of exposure 10–15 seconds; other conditions as in Table I.

Solvent	pH of solvent	Total molar	s_{20} H	s_{20} K	
NaAc, HAc, NaCl	3.6	0.20	17.3		
" " "	4.0	0.20	17.4		
" " "	4.6	0.20	16.6		
" " "	5.0	0.20	16.7		
" " "	5.5	0.20	16.2		
Na_2HPO_4, KH_2PO_4, NaCl	6.0	0.20	16.8		
" " "	6.8	0.20	15.6		
" " "	6.8	0.20	16.6		
" " "	7.4	0.20	16.3		
" " "	8.0	0.20	15.6		
$Na_2B_4O_7$, KH_2PO_4, NaCl	8.5	0.20	15.7		
" " "	9.0	0.20	15.7		
" " "	9.4	0.20	16.8	+	
" Na_2CO_3, NaCl	9.7	0.20	17.3	5.40	
" " "	10.0	0.20	16.9	5.61	
" " "	10.1	0.20	17.1	3.89	
" " "	10.1	0.20	15.2	5.68	
" " "	10.3	0.20	16.7	5.81	
" " "	10.6	0.20	15.7	5.32	
Na_2HPO_4, NaOH, NaCl	10.8	0.30		5.47	Inhomogeneous
" " "	11.3			3.83*	Inhomogeneous
			16.4	4.10	

* Not used for the calculation of mean value.

extent into particles with the sedimentation constant 4.10 (comp. K). The low-molecular fraction increases with pH. At 10.8 H has disappeared. K is rather inhomogeneous and becomes more so in the more alkaline solutions. Figures 2 and 3 give examples of sedimentation runs and Fig. 4 (Plate I) the pH-stability diagram.

Nephrops norvegicus

Kristineberg, Sweden

The main component G of the hemocyanin is present alone in the blood in the pH-region 4.0–8.0. It is homogeneous with regard to molecular weight and has the sedimentation constant 24.5. At pH 8.0 G is partly dissociated into component H with the sedimentation constant 17.1. Dissociation goes further at pH 10.2 and component K with sedimentation constant 5.97 is formed. Thus in the pH-region 8.0–10.2 the hemocyanin consists of the components G and H, in the

TABLE III

Nephrops norvegicus

Dilution of blood, 10 times; centrifugal force, 150,000 (speed 45,000 r.p.m.); in the last run 220,000 (speed 56,000 r.p.m.); other conditions as in Table I.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	s_{20} K	
HAc, NaAc, NaCl.....	3.6	0.22		17.6		Very inhomogeneous
" " "	4.0	0.22	23.9			Slightly inhomogeneous
" " "	4.5	0.22	24.0			
" " "	5.0	0.22	24.6			
" " "	5.5	0.22	23.4			
KH_2PO_4, Na_2HPO_4, NaCl	6.0	0.22	25.8			
" " "	6.5	0.22	25.1			
" " "	6.8	0.22	23.9			NiO-filter
" " "	6.8	0.22	25.5			
" " "	7.4	0.22	25.1			
" " "	7.7	0.22	24.3			
" " "	8.0	0.22	25.1	+		
KH_2PO_4, $Na_2B_4O_7$, NaCl.	8.5	0.27	23.5	+		
Na_2CO_3, $Na_2B_4O_7$, NaCl.	9.5	0.25	24.1	16.7		
" " " .	10.0	0.25	24.3	+		
" " " .	10.2	0.25	23.6*	18.6	5.98	
" " " .	10.5	0.25	22.5*	17.7	6.46	
" " " .	10.7	0.25	19.1*	16.6	5.92	
Na_2HPO_4, NaOH, NaCl.	11.0	0.30	19.9*	15.8	5.42	
" " " .	11.4	0.28			6.08	Containing inhomogeneous component with $s_{20} = 3.54$
" " " .	11.7	0.28			8.95	Very inhomogeneous
			24.5	17.1	5.97	

* Not used for calculation of the mean value.

pH-region 10.2–11.4 of G, H, and K. The decreasing of the sedimentation constant of G in the latter region is probably due to a change in shape of the molecule. At 11.4 G and H are both completely dis-

sociated and a still more low-molecular product appears with the sedimentation constant 3.5. At pH 11.7 the material is completely inhomogeneous. Figure 5 (Plate I) gives the pH-stability diagram.

TABLE IV

Homarus vulgaris

Dilution of blood 6–10 times; centrifugal force 120,000–220,000 (speed 40,000–55,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	s_{20} K	
Undiluted blood			13.5*			High viscosity
Diluted 3 times 0.1-mKCl			21.0*			High viscosity
HCl, Na-citrate, NaCl	3.4		23.3			
HAc, NaAc, KCl	4.0	0.12	24.5			Purified hemocyanin (Am_2SO_4-prec.)
HAc, NaAc, NaCl	4.0	0.20	22.8			
HAc, NaAc, KCl	5.0	0.12	22.6			
HAc, NaAc, NaCl	5.0	0.20	23.4			
KH_2PO_4, Na_2HPO_4, NaCl	6.0	0.20	22.5			
" " "	6.8	0.20	23.6			
KH_2PO_4, Na_2HPO_4, KCl	6.8	0.12	20.7			
" " "	8.0	0.12	22.6			
KH_2PO_4, $Na_2B_4O_7$, NaCl	9.0	0.20	21.9			
NaOH, Na_2HPO_4, KCl	9.8	0.15	23.4	16.9		
$Na_2B_4O_7$, Na_2CO_3, NaCl	10.0	0.25		17.3		
" " "	10.1	0.23		14.8		
" " "	10.0	0.20	22.2			
NaOH, Na_2HPO_4, NaCl	10.2	0.05	19.3	15.5		
$Na_2B_4O_7$, Na_2CO_3, NaCl	10.3	0.20		15.5		
" " "	10.3	0.20	21.8			
" " "	10.6	0.17		16.0	+	
NaOH, Na_2HPO_4, NaCl	10.7	0.06		15.6	6.64	
$Na_2B_4O_7$, Na_2CO_3, NaCl	10.5	0.20	21.5*			
" " "	10.3	0.20	19.7*			
" " "	10.7	0.20	19.8*	16.8		
Na_2CO_3, $Na_2B_4O_7$, NaCl	10.8	0.20		16.6		
NaOH, Na_2HPO_4, NaCl	11.0	0.06		13.8*	+	
" " "	11.0	0.32		12.2*	9.72	Inhomogeneous
" " "	11.4	0.07			5.81	Inhomogeneous
			22.6	16.1		

* Not used for calculation of mean value.

Homarus vulgaris

Havstensund, Sweden

The sedimentation constant of the main component G is 22.8. G is present alone in the blood from pH 3.4 to 9.8. At pH 9.8 and

above a dissociation product, H, is formed with the sedimentation constant 16.1. When both G and H are present together it is difficult to determine their separate sedimentation constants. Generally G is observed in solutions of pH up to around 10.5 and H up to 11. At pH 11.4 the sedimentation picture shows only low-molecular inhomogeneous material. Figure 6 (Plate I) gives the pH-stability diagram.

Astacus fluviatilis

Upland, Sweden

The hemocyanin shows more than one component in the whole pH-range investigated. In the pH region 3.6–10, 80–85 per cent of the

TABLE V

Astacus fluviatilis

Dilution of blood 10 times; centrifugal force 150,000 (speed 45,000 r.p.m.); for the last three runs 250,000 (speed 59,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	s_{20} K	
HAc, NaAc, NaCl	3.6	0.20	23.2	17.5		Inhomogeneous
" " "	4.0	0.20	23.4	15.4		
" " "	4.6	0.20	23.1	15.1		
" " "	5.0	0.20	24.2	16.7		
KH_2PO_4, Na_2HPO_4, NaCl	5.6	0.20	23.1	16.4		
" " "	6.2	0.20	22.3	15.7		
" " "	6.8	0.20	22.3	14.8		
" " "	7.2	0.20	23.1	16.7		
" " "	7.7	0.20	24.5	16.6		
" " "	8.0	0.20	23.7	16.7		
KH_2PO_4, $Na_2B_4O_7$, NaCl	8.2	0.25	21.8	16.1		
" " "	8.5	0.25	23.6	16.3		
" " "	9.0	0.25	23.7	16.1		
Na_2CO_3, $Na_2B_4O_7$, NaCl	9.4	0.25	24.0	17.5		
" " "	9.7	0.25	22.1	15.4		
" " "	10.2	0.25	24.1	16.6		
" " "	10.3	0.23	24.4	17.3	3.73	
" " "	10.6	0.21	22.9	15.6	2.37	
NaOH, Na_2HPO_4, NaCl	10.9	0.27		12.5*	5.15	
" " "	11.2	0.27			4.65	Inhomogeneous
" " "	11.5	0.27			4.19	"
			23.3	16.3	4.02	

* Not used for calculation of mean value.

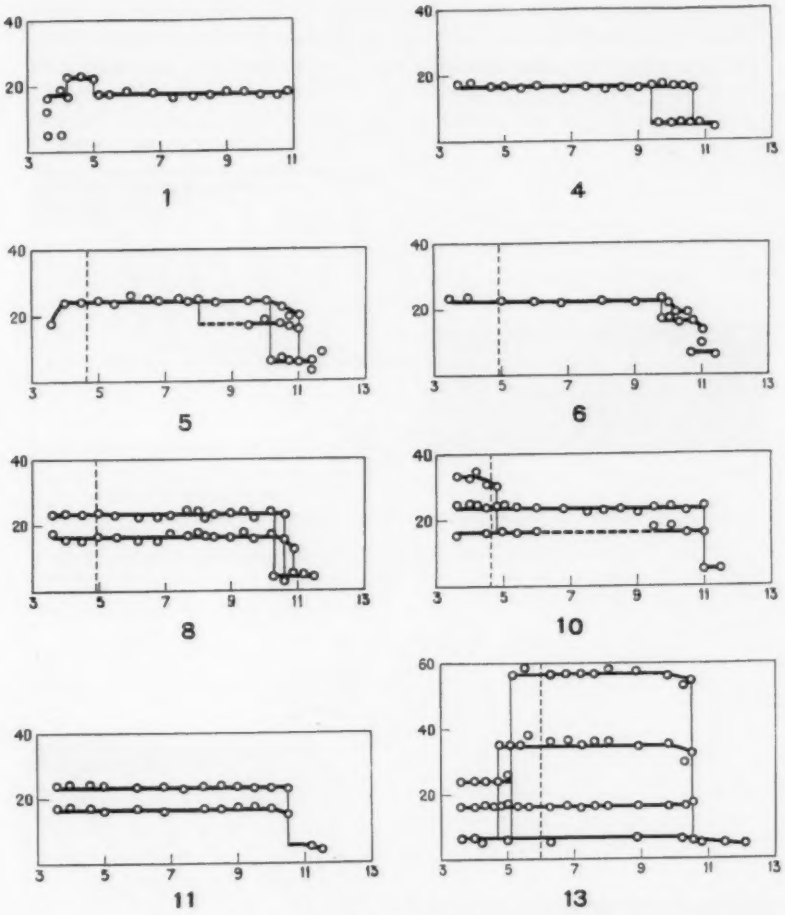


PLATE I

pH-stability diagrams for hemocyanins of species listed below. Abscissæ in all figures, pH; ordinates, s_{20}. The dotted lines in Figs. 5, 6, 8, 10 and 13 indicate the positions of the isoelectric points.

FIG. 1. *Pandalus borealis.*
FIG. 4. *Palinurus vulgaris.*
FIG. 5. *Nephrops norvegicus.*
FIG. 6. *Homarus vulgaris.*
FIG. 8. *Astacus fluviatilis.*
FIG. 10. *Cancer pagurus.*
FIG. 11. *Carcinus mænas.*
FIG. 13. *Limulus polyphemus.*

protein has the sedimentation constant 23.3 (component G). The other component present, H, has the sedimentation constant 16.3. Around pH 10 the amount of H starts to increase, G to decrease. At

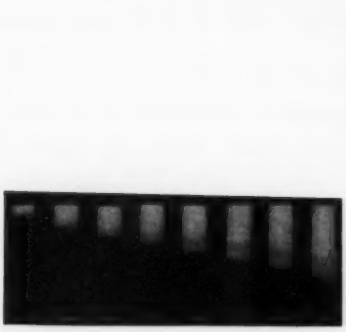


FIG. 7*a*.

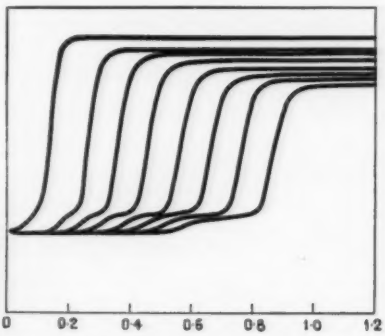


FIG. 7*b*.

FIG. 7. Sedimentation pictures (*a*) with photometric records (*b*) for hemocyanin from *Astacus fluviatilis* at pH 7.7 (s_{20} = 23.3 and 16.3); centrifugal force 150,000; time between exposures 5 minutes.

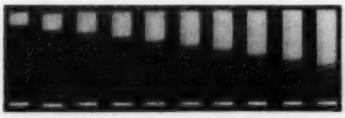


FIG. 9*a*.

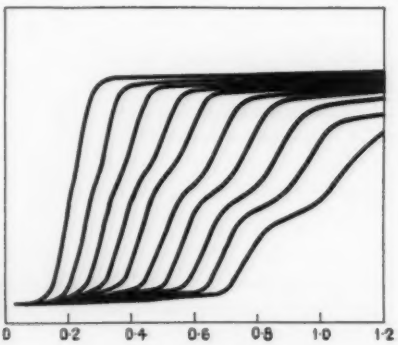


FIG. 9*b*.

FIG. 9. Sedimentation pictures (*a*) with photometric records (*b*) for the hemocyanin from *Cancer pagurus* at pH 4.2 (s_{20} = 32.7, 23.6, and 16.4); centrifugal force 120,000; time between exposures 5 minutes.

pH 10.3 a low molecular component, K, is formed. In a narrow pH-range, 10.3–10.6 G, H, and K appear together. At pH 10.9 G has totally disappeared and there is very little left of H. In more alkaline buffers all of the material is low-molecular and inhomogeneous. Figure 7 gives an example of a sedimentation run and Fig. 8 (Plate I) the pH-stability diagram.

Cancer pagurus

Havstensund, Sweden

The main component, G, of this blood has the sedimentation constant 23.6 and is observed in the wide pH-range 3.6–11.0. G is always accompanied by another component H with the sedimentation constant 16.4. H is present only in very small quantity. In the pH range 3.6–4.8 appears a third component, F, aggregation product of G,

TABLE VI

Cancer pagurus

Dilution of blood 6 times; centrifugal field 115,000 (speed 40,000 r.p.m.); in the last run 220,000 (55,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} F	s_{20} G	s_{20} H	s_{20} K	
HCl Na-citrate, NaCl....	3.0	0.20					Aggregated and inhomogeneous
HAc, NaAc, NaCl.....	3.6	0.20	32.7	24.7	15.3		
" " "	4.0	0.20	32.5	24.9	+		
" " "	4.2	0.20	34.6	25.0	+		
" " "	4.5	0.20	31.0	24.2	16.0		
" " "	4.8	0.20	29.9*	24.1	+		
" " "	5.0	0.20		23.8	16.5		
" " "	5.4	0.20		23.8	16.1		
KH_2PO_4, Na_2HPO_4, NaCl.	6.0	0.20		23.7	16.6		
" " " .	6.8	0.20		23.2	+		
" " " .	7.5	0.20		22.5	+		
KH_2PO_4, $Na_2B_4O_7$, NaCl..	8.0	0.25		22.7	+		
" " " ..	8.5	0.25		23.1	+		
Na_2CO_3, $Na_2B_4O_7$, NaCl..	9.0	0.25		22.1	+		
" " " ..	9.5	0.25		23.7	17.5		
" " " ..	10.0	0.25		24.3	17.7		
" " " ..	10.5	0.25		22.7	15.7		
" " " ..	10.5	0.25		23.0	+		
Na_2HPO_4, NaOH, NaCl..	11.0	0.30		23.9	16.2	4.60	Inhomogeneous
" " " ..	11.5	0.30				4.82	"
			32.7	23.6	16.4	4.71	

* Not used for calculation of mean value.

with the sedimentation constant 32.7. At pH 11.0 component K with sedimentation constant 4.77 is observed together with G and H. At

pH 11.5 G and H have disappeared. K is not quite homogeneous. At pH 3.0 the hemocyanin is aggregated and non-uniform. Figure 9 gives an example of a sedimentation run and Fig. 10 (Plate I) the pH-stability diagram.

TABLE VII

Carcinus mænas

Dilution of blood 6 times; centrifugal force 175,000 (speed 50,000 r.p.m.); in the last two runs 280,000 (speed 63,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} G	s_{20} H	s_{20} K	
HAc, NaAc, NaCl	3.6	0.20	23.6	17.1		
" " "	4.0	0.20	24.0	17.1		
" " "	4.6	0.20	23.9	16.8		
" " "	5.0	0.35	22.5	15.7		
KH_2PO_4, Na_2HPO_4, NaCl	6.0	0.35	23.0	16.5		
" " KCl	6.8	0.12	23.5	16.2		
" " NaCl	7.4	0.20	22.6	+		
" " KCl	8.0	0.12	23.4	16.6		
" $Na_2B_4O_7$, NaCl	8.5	0.25	23.7	16.3		
" " "	9.0	0.25	23.7	17.3		
" " "	9.5	0.25	23.1	17.3		
" " "	10.0	0.25	22.7	16.7		
" " "	10.5	0.25	22.5	14.5*		
NaOH " "	11.2	0.27			5.00	Inhomogeneous
" " "	11.5	0.30			4.17	Inhomogeneous
			23.3	16.7		

* Not used for calculation of mean value.

Carcinus mænas

Kristineberg, Sweden

The sedimentation constant of the main component G is 23.3. It is stable from pH 3.6–10.5. A smaller component with the sedimentation constant 16.7 is present throughout the same range but to a very small amount. At pH 11.2 both are dissociated into component K, a low-molecular inhomogeneous split-product. Figure 11 (Plate I) gives the pH-stability diagram.

Arachnomorpha: Xiphosura:

Limulus polyphemus

Woods Hole, Mass., U. S. A.

In the blood four different components are present at the same time

in the pH-range 5.2–10.5. Their sedimentation constants are 56.6 (D), 34.6 (F), 16.1 (H), and 5.87 (K). The proportions between their concentrations are unchanged inside this region. Above 10.5 D, F, and H disappear; they split up into K. Below pH 5.2 D and F dis-

TABLE VIII

Limulus polyphemus

Dilution of blood 4 times; centrifugal force 125,000 (speed 41,000 r.p.m.); in the last three runs 300,000 (speed 65,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} D	s_{20} F	s_{20} G	s_{20} H	s_{20} K	
HAc, NaAc, NaCl.....	3.6	0.36			24.2	16.2	6.02	
" " "	4.0	0.19			23.5	16.1	6.15	
" " "	4.2	0.36			23.5	16.4	5.24	
" " "	4.4	0.19			23.3	16.2	+	
" " "	4.6	0.19	55.2*		23.5	15.8	+	
" " "	4.6	0.19			24.5	16.5	+	
" " "	4.8	0.19		35.2	23.1	15.8	+	
" " "	4.8	0.19			24.7	17.0	+	
" " "	5.0	0.19			25.8	16.8	6.3	
" " "	5.2	0.19	56.3	34.8		16.1	+	
KH_3PO_4, Na_2HPO_4, NaCl	5.4	0.24	58.2	35.3		17.2	+	
" " "	5.6	0.19	59.7	38.1		16.2	+	
" " "	6.3	0.36	56.5	36.7		15.5	+	
" " "	6.3	0.36	+	+		15.5	5.0†	
" " "	6.8	0.19	57.3	36.4		16.3	+	
" " "	7.2	0.36	57.1	35.1		15.5	+	
" " "	7.6	0.19	56.4	36.0		15.9	+	
" " "	8.0	0.36	58.0	36.0		16.0	+	
Na_2HPO_4, NaCl.....	8.9	0.20	58.2	33.8		16.1	6.28	
" NaOH, NaCl .	9.8	0.22	56.1	35.1		15.8	+	
" " " .	10.2	0.22	52.6	30.0		17.6	6.25	
Na_2CO_3, $Na_2B_4O_7$, NaCl .	10.3	0.22	53.4	29.6		14.4	5.70	
" " " .	10.5	0.23	54.6	32.3		16.1	5.86	
Na_2HPO_4, NaOH, NaCl .	10.7	0.23					5.14	
Na_2CO_3, $Na_2B_4O_7$, NaCl .	10.8	0.23				+	4.88*	
Na_2HPO_4, NaOH, NaCl .	11.5	0.27					4.45*	Dialysed blood
Na_2HPO_4, NaOH, NaCl .	12.1	0.27					4.26*	Dialysed blood
			56.5	34.6	24.0	16.1	5.87	

* Not used for the calculation of mean value.

† Made at 60,000 r.p.m. and with blood diluted 2 times to get component K.

sociate in two ways. The concentration of K apparently increases; *i.e.*, some part of them must form more of K. A new component, G, with the sedimentation constant 24.0 appears in a rather high concentration; and G must be a dissociation product of D and F as the concentration of H does not change and the concentration of K increases.

Figure 12 gives an example of a sedimentation run and Fig. 13 (Plate I) the pH-stability diagram.

Mollusca

The bloods of the Amphineura, the Gastropoda and the Cephalopoda contain hemocyanin. Because of the difficulty of obtaining sufficient material from specimens of the first class our investigation has been limited to species belonging to the two latter classes.

Conchifera: Gastropoda:

Blood from the following animals belonging to this group has been investigated: *Littorina littorea*, *Neptunea antiqua*, *Buccinum undatum*,

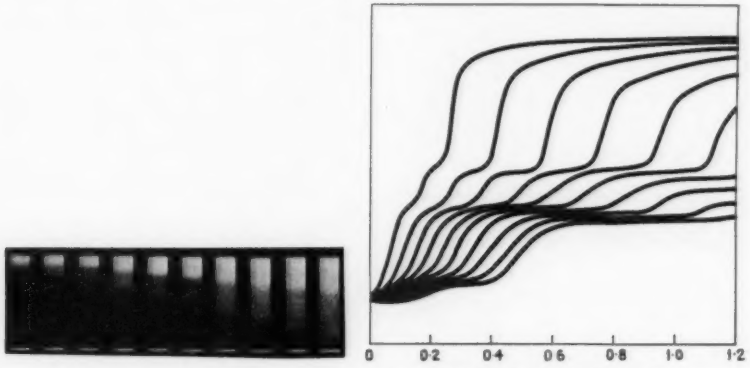


FIG. 12*a*. FIG. 12*b*.

FIG. 12. Sedimentation pictures (*a*) with photometric records (*b*) for the hemocyanin from *Limulus polyphemus* at pH 6.8 (s_{20} = 56.6, 34.6, 16.1, and 5.87); centrifugal force 120,000; time between exposures 5 minutes.

Busycon canaliculatum, *Helix pomatia*, *Helix arbustorum*, *Helix nemoralis*, *Helix hortensis*, *Limax maximus*. They show extremely varied stability diagrams and a great number of dissociation products. The main component has a sedimentation constant of about 100. In blood from two of the animals, *Busycon* and *Limax*, a higher component with the sedimentation constant near 130 is found. All, except *Neptunea*, show a dissociation product with a sedimentation constant of about 60. Very often a component with sedimentation constant of approximately 16 is found and the final alkaline dissociation products have sedimentation constants around 11. It is also striking that the four kinds of *Helix* have different stability diagrams, so that each of them could be characterized thereby.

Littorina littorea

Kristineberg, Sweden

Between pH 5 and 8.5 this hemocyanin shows one homogeneous component B, with the sedimentation constant 99.7. At pH 8.5 B is partly split up into components C and H. In the pH-range 8.5–10.7 most of the hemocyanin is present in form of C, with the sedimentation constant 63.3. H appears only in very small amount up to pH 10.8

TABLE IX

Littorina littorea

Dilution of blood 4 times; centrifugal force 49,000 (speed 25,000 r.p.m.); in the last three runs 180,000 (speed 50,000 r.p.m.); thickness of column of solution, 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	
NaAc, HAc, NaCl.....	4.0	0.22				Solubility too low for determination
" " "	5.0	0.22	96.9			
" " "	5.5	0.22	99.7			
Na_2HPO_4, KH_2PO_4, NaCl	5.9	0.22	99.8			
" " "	6.2	0.22	98.4			
" " "	6.8	0.22	98.5			
" " "	7.4	0.22	97.0			
" " "	8.0	0.22	94.6			
$Na_2B_4O_7$, " "	8.5	0.27	102.1	63.5	+	
" " "	9.0	0.27	102.1	64.8	+	
" Na_2CO_3, "	9.5	0.25	101.4	63.0	+	
" " "	10.0	0.25	100.0	62.2	+	
" " "	10.3	0.25	97.9	62.5	+	
" " "	10.5	0.25	104.6	64.3	+	
" " "	10.6	0.25	102.0	62.3	+	
" " "	10.7	0.25	102.9	63.4	+	
" " "	10.8	0.25	97.3	59.5*	14.5	
Na_2HPO_4, NaOH, "	11.1	0.25			15.6	
" " "	11.3	0.25			11.3*	
" " "	11.5	0.25			12.9*	
			99.7	63.3	15.0	

* Not used for the calculation of mean value.

where most of B and C are dissociated into H and above it is the only component present. When first observed H has a sedimentation constant of about 15, but it decreases towards the alkaline side and the protein becomes inhomogeneous. At pH 4.0 precipitation takes place. Figure 14 (Plate II) gives the pH-stability diagram.

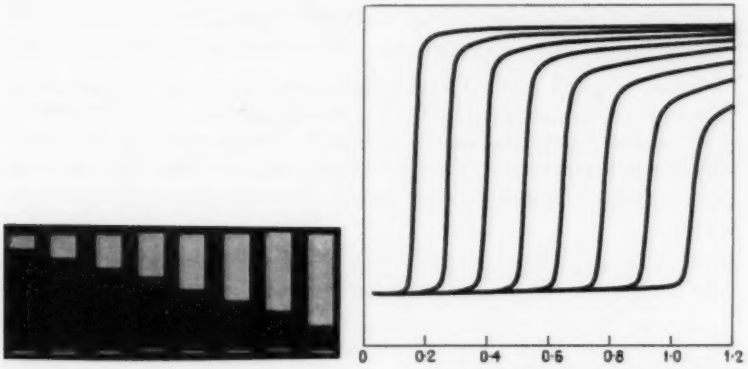


FIG. 15*a*. FIG. 15*b*.

FIG. 15. Sedimentation pictures (*a*) with photometric records (*b*) for the hemocyanin from *Neptunea antiqua* at pH 8.0 (s_{20} = 104.1); centrifugal force 40,000; time between exposures 5 minutes.

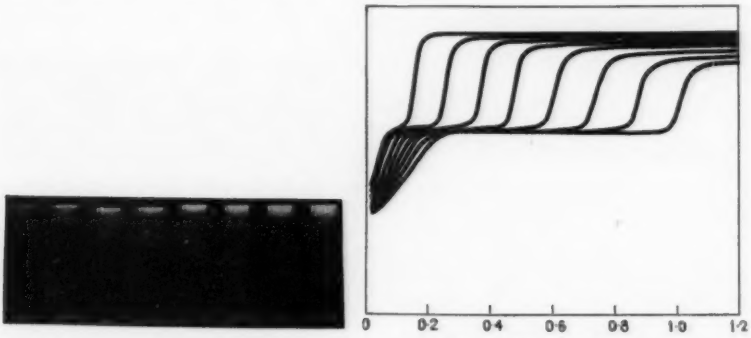


FIG. 16*a*. FIG. 16*b*.

FIG. 16. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Neptunea antiqua* at pH 8.6 (s_{20} = 104.1 and 14.3); centrifugal force 60,000; time between exposures 5 minutes.

Neptunea antiqua

Kristineberg, Sweden

In the pH-range 4.6–8.6 the hemocyanin is homogeneous and shows one component, B, the sedimentation constant of which is 104.1. At 8.6 there begins a partial splitting into component H with the sedimentation constant 14.3. In the pH-region 8.6–10.0 B and H are

present together. At pH 10.8 the splitting is total, B disappears and a new component, I, with a sedimentation constant near 13 appears. The sedimentation constant of I decreases at a higher pH and the protein becomes inhomogeneous. In acid solutions aggregation takes place. The material becomes more and more inhomogeneous, the particles larger. At pH 3.0 most of the protein is precipitated. Figures 15 and 16 give examples of sedimentation runs and Fig. 17 (Plate II) the pH-stability diagram.

TABLE X

Neptunea antiqua

Dilution of blood 15–20 times; centrifugal force 49,000 (speed 25,000 r.p.m.); in the last three runs 180,000 (speed 50,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} H	s_{20} I	
NaAc, HAc, NaCl.....	3.0	0.19				Most of the material precipitated
" " "	3.6	0.19	116.8*			Inhomogeneous
" " "	4.0	0.19	138.2*			"
" " "	4.2	0.19	110.6*			"
" " "	4.6	0.19	102.4			
" " "	5.0	0.19	104.7			
Na_2HPO_4, KH_2PO_4, NaCl .	5.5	0.19	102.7			
" " " .	6.0	0.19	101.1			
" " KCl . .	6.8	0.14	106.7			
" " NaCl .	7.1	0.19	102.8			
" " " .	7.5	0.19	105.6			
" " " .	8.0	0.19	106.9			
$Na_2B_4O_7$, " " .	8.6	0.20	100.7	14.0		
" " " .	8.8	0.20	104.0	11.6		
" " " .	9.0	0.20	101.4	13.6		
" Na_2CO_3, " .	9.25	0.20	107.8	+		
" " " .	9.5	0.20	102.8	+		
" " " .	10.0	0.20	107.0	15.9		
" " " .	10.8	0.20		15.7	13.8	Inhomogeneous
" " " .	10.8	0.20		15.1	13.3	"
NaOH, Na_2HPO_4, " .	12.1	0.27			9.48	"
			104.0	14.3		

* Not used for calculation of mean value.

Buccinum undatum

Kristineberg, Sweden

The main component, B, of this hemocyanin is observed in the pH-range 5–10.8. Its sedimentation constant is 102.1. B is present alone from pH 5 to 9.5. Here a very small quantity of C, a component

with sedimentation constant 63.8 is formed. The relative amount of C increases with pH. Above 10.8 B and C are dissociated into more inhomogeneous material with a sedimentation constant around 11. At pH 4.0 the hemocyanin is precipitated. Figure 18 (Plate II) gives the pH-stability curve.

TABLE XI

Buccinum undatum

Dilution of blood 7 times; centrifugal force 49,000 (speed 25,000 r.p.m.); in the last four runs 220,000 (speed 55,000 r.p.m.); other conditions as in Table II.

Solvent			pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} I	
		NaCl..		0.17	104.5			
			4.0					Protein precipitated
HAc,	NaAc,	" ..	5.0	0.20	104.0			
KH_2PO_4,	Na_2HPO_4,	" ..	5.9	0.20	98.4			
"	"	" ..	6.2	0.20	100.9			
"	"	KCl...	6.8	0.12	102.7			
"	"	NaCl..	7.4	0.20	100.7			
"	$Na_2B_4O_7$,	KCl...	8.0	0.12	103.9			
"	"	NaCl..	8.5	0.25	103.2			
"	"	" ..	9.0	0.25	99.6			
Na_2CO_3,	"	" ..	9.5	0.25	101.5			
"	"	" ..	10.0	0.25	105.6	64.6		
"	"	" ..	10.8	0.25	100.5	63.1	+	
"	"	" ..	10.8	0.25			12.8	
NaOH,	Na_2HPO_4,	" ..	11.3	0.30			10.5	Very inhomogeneous
"	"	" ..	11.5	0.30			11.0	" "
"	"	" ..	12.1	0.35			9.2	" "
					102.1	63.8		

Busycon canaliculatum

Woods Hole, Mass., U.S.A.

The hemocyanin from this animal shows a very complicated stability diagram. Four well-defined components, A, B, C, and H are observed within the investigated pH-range 3–12. The sedimentation constants are: for A 130.4, for B 101.7, for C 61.1 and for H 13.5. A and B are present together in the region 4.5–7.7. The relative amount of A is larger towards the acid side. It is not perfectly homogeneous; the sedimentation curves have a shape indicating the presence of slightly faster sedimenting particles. At 4.0 the protein aggregates, and becomes inhomogeneous. In the region 7.7–9.0 there

are three components present, B, C and H. The amount of B is larger in the less alkaline part of the region, at 9.0 it is only several per cent of the whole protein. The percentages of C and H increase with pH. In the pH-region 9.0–10.8 the components C and H are found. Above 10.8 only H is left and it there becomes more and

FIG. 19*a*.

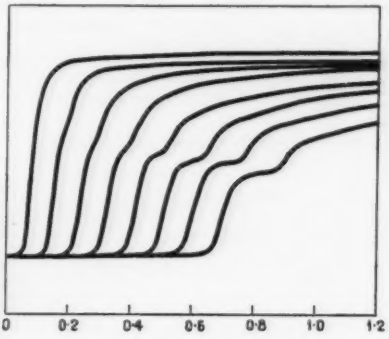


FIG. 19*b*.

FIG. 19. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Busycon canaliculatum* at pH 6.5 (s_{20} = 130.4 and 101.7); centrifugal force 40,000; time between exposures 5 minutes.

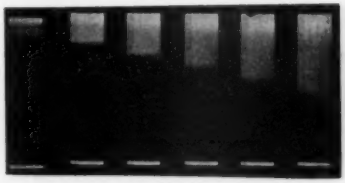


FIG. 20*a*.

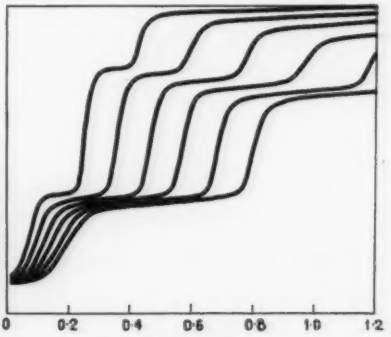


FIG. 20*b*.

FIG. 20. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Busycon canaliculatum* at pH 8.5 (s_{20} = 101.7, 61.1, and 13.5); centrifugal force 70,000; time between exposures 5 minutes.

more inhomogeneous. Figures 19 and 20 give examples of sedimentation runs and Fig. 21 (Plate II) the pH-stability curve.

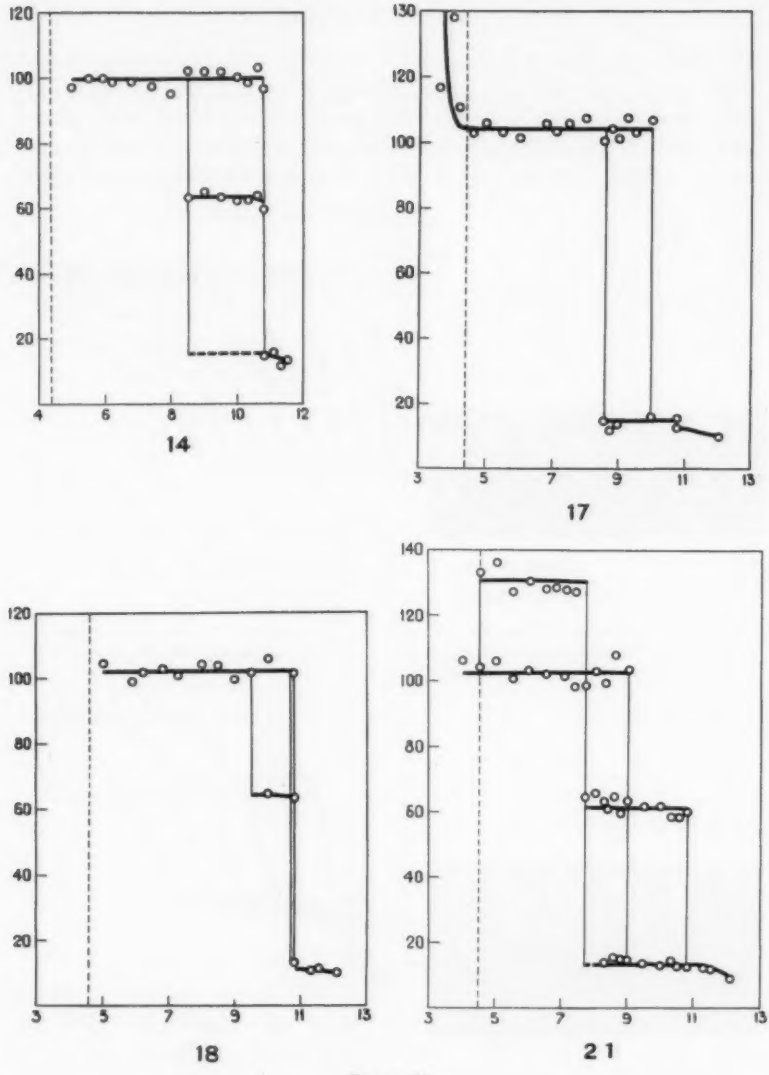


PLATE II

pH-stability diagrams for hemocyanins of species listed below. Abscissæ in all figures, pH; ordinates, s_{20}. The dotted lines indicate the positions of the isoelectric points.

FIG. 14. *Littorina littorea.*
FIG. 17. *Neptunea antiqua.*
FIG. 18. *Buccinum undatum.*
FIG. 21. *Busycon canaliculatum.*

Helix pomatia

Upsala, Sweden

The hemocyanin appears in four different well-defined molecular species, B with the sedimentation constant 98.9, C with 62.0, H with

TABLE XII

Busycon canaliculatum

Dilution of blood 20 times, centrifugal force in the pH-range 3–8, 35,000 (speed 23,000 r.p.m.), in the pH-range 8–11, 60,000 (speed 30,000 r.p.m.), above pH 11, 200,000 (speed 55,000 r.p.m.); thickness of column of solution, 0.6 cm., other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} A	s_{20} B	s_{20} C	s_{20} I	
HCl, Na-citrate, NaCl...	3.0	0.30		45.5			Inhomogeneous
HAc, NaAc, NaCl.....	3.4–3.6	0.19					Solubility too
" " "	4.0	0.19		106.0			low for deter-
" " "	4.5	0.19	133.0	103.8			mination
" " "	5.0	0.19	137.0	107.2			
" " "	5.0	0.19	134.9	104.6			
KH_2PO_4, Na_2HPO_4, NaCl	5.5	0.19	126.8	100.2			
" " "	6.0	0.19	130.1	102.8			
" " "	6.5	0.19	128.1	102.1			
" " KCl .	6.8	0.12	128.5	102.0			
" " NaCl	7.1	0.19	127.7	101.7			
" " "	7.4	0.19	127.3	98.3			
" " "	7.7	0.19		98.6	64.1	+	
" " KCl .	8.0	0.12		102.9	65.5	+	
" $Na_2B_4O_7$, NaCl	8.3	0.23		98.9	62.7	+	
" " "	8.4	0.23		96.1	59.6	12.2	
" " "	8.4	0.23		102.2	60.3	14.1	
" " "	8.5	0.23		97.1	60.0	14.2	
" " "	8.6	0.23		107.4	64.0	14.9	
Na_2CO_3, " "	9.0	0.23		103.3	61.7	14.7	
" " "	9.5	0.23			60.9	13.0	
" " "	10.0	0.23			60.5	13.0	
" " "	10.3	0.23			58.0	14.0	
" " "	10.5	0.23			58.2	12.6	
" " "	10.8	0.23			58.5	13.6	
Na_3PHO_4, NaOH, "	11.3	0.27				12.0	
" " "	11.5	0.30				11.5*	Inhomogeneous
" " "	12.1	0.30				8.52*	"
			130.4	101.7	61.1	13.5	

* Not used for calculation of mean value.

16.0, and I with 12.1. The main component is B, present in solutions with a pH from 3.6 to 8.2. It is alone in the pH-region 4.6–7.4 and accompanied by C in the regions 3.6–4.6 and 7.4–8.2. H is present in very small quantity together with B and C in the pH-range 7.9–8.2,

TABLE XIII

Helix pomatia

Dilution of blood 15 times; centrifugal force in the range pH 3–8, 50,000 (speed 27,000 r.p.m.); above pH 8 150,000 (speed 45,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	s_{20} I	
HCl, Na-citrate, NaCl..	3.3	0.27					22.5 Inhomogeneous
HAc, NaAc, NaCl.....	3.6	0.20	98.2	61.9			
" " "	3.6	0.37		60.5			26.5 "
" " "	3.8	0.37	102.5	63.1			
" " "	3.8	0.20	95.6	59.2			
" " "	4.0	0.20	100.7	59.3			
" " "	4.0	0.20	97.6	62.6			
" " "	4.2	0.20	103.1	61.8			
" " "	4.2	0.37	102.6	66.2			
" " "	4.6	0.20	95.9	+			
" " "	5.0	0.20	102.2				
" " "	5.4	0.20	99.8				
KH_2PO_4, Na_2HPO_4, NaCl	5.5	0.20	102.5				
" " "	6.0	0.20	94.8				
" " "	6.4	0.20	102.1				
" " "	7.0	0.20	98.3				
" " "	7.4	0.20	94.3	+			
" " "	7.5	0.20	89.6*	+			Bad separation of the 2 components
" " "	7.6	0.20	102.6	56.7			
" " "	7.7	0.20	97.4	64.2			
" " "	7.9	0.20	96.5	63.0			
" " "	8.0	0.20	97.3	62.5	+		
" $Na_2B_4O_7$, "	8.1	0.23	94.1	65.8			
" " "	8.2	0.23	99.3	62.8	19.5*		
" " "	8.3	0.23			16.5		
" " "	8.4	0.24			15.3		
" " "	8.5	0.24			16.5		
" " "	8.6	0.24			16.6		
" " "	8.9	0.24			17.7		
" " "	9.3	0.24			15.7	+	
Na_2CO_3, " "	9.4	0.22			15.8	13.4	
" " "	9.5	0.23			15.7	11.9	
" " "	9.7	0.23			15.4	+	
" " "	9.8	0.22			14.8		
" " "	9.8	0.23			14.7	11.9	
" " "	9.8	0.23			15.6	11.1	
" " "	10.0	0.23			18.1	+	
" " "	10.3	0.23			15.0	11.5	
" " "	10.5	0.23			15.1	11.7	
" " "	10.6	0.42			18.1	13.9	
" " "	10.8	0.25			+	11.3	
NaOH, Na_2HPO_4, "	11.5	0.30				9.9*	Inhomogeneous
" " "	12.1	0.30				8.4*	"
			98.9	62.0	16.0	12.1	

* Not used for the calculation of mean value.

alone between pH 8.2 and 9.3, and together with I in the pH-range 9.3–10.8. In more alkaline solutions the hemocyanin is inhomogeneous and the sedimentation constant decreases. Below pH 3.6 the material is non-uniform and the sedimentation constants are irregular but apparently decreasing. Figure 22 (Plate III) gives the pH-stability diagram.

TABLE XIV

Helix arbustorum

Dilution of blood 5 to 30 times; centrifugal force in the pH-region 3–8, 50,000 (speed 26,000 r.p.m.); above pH 8 175,000 (speed 50,000 r.p.m.); thickness of column of solution 0.4–2.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	s_{20} I	
HCl, Na-citrate, NaCl....	2.5	0.20					Very inhomogeneous
" " "	2.9	0.20			20.9	12.5	" "
HAc, NaAc, NaCl.....	3.6	0.20	83.8	56.0			
" " "	4.0	0.20	94.2	56.5			
" " "	4.6	0.20	89.7	71.5			
" " "	5.0	0.20	95.4	+			
" " "	5.0	0.20	87.8	59.8			
KH_2PO_4, Na_2HPO_4, NaCl.	5.6	0.20	97.0	74.1			
" " " .	6.0	0.20	92.2	65.2			
" " " .	6.3	0.20	90.0	61.9			
" " " .	6.5	0.20	90.1	68.9			
" " " .	6.8	0.20	88.3	63.0			
" " " .	7.0	0.20	90.8	+			
" " " .	7.4	0.20	93.1	66.5			
" " " .	7.7	0.20	96.8	60.9			
" " " .	8.0	0.20	87.2	+			
" $Na_2B_4O_7$, NaCl..	8.2	0.25			16.1		
" " " ..	8.5	0.25			16.1		
" " " ..	8.7	0.25			15.6	+	
Na_2CO_3, " " ..	9.0				16.4	13.0	
" " " ..	9.4				14.9	11.0	
" " " ..	9.5				16.8	12.5	
" " " ..	10.14	0.25			15.5	11.9	
" " " ..	10.3				14.0	10.9	
" " " ..	10.6				10.9	9.7	
Na_2HPO_4, NaOH, NaCl..	10.8	0.27			15.7	11.3	
" " " ..	11.0	0.32			13.7	10.4	
" " " ..	11.3	0.27			14.6	9.3	
			91.2	64.1	15.0	11.1	

Helix arbustorum

Upsala, Sweden

The hemocyanin has two components, B and C, in the pH-range 3.6–8.0. The two components seem to influence each other in a way

that makes accurate determination of their sedimentation constants rather difficult. The mean value for B is 91.2 and for C 64.1.

The proportions between B and C change in an irregular way.

FIG. 23*a*.

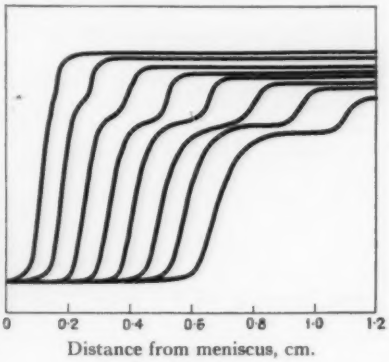


FIG. 23*b*.

FIG. 23. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Helix arbustorum* at pH 7.7 (s_{20} = 91.2 and 64.1); centrifugal force 50,000; time between exposures 5 minutes.

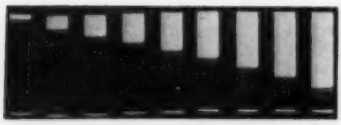


FIG. 25*a*.

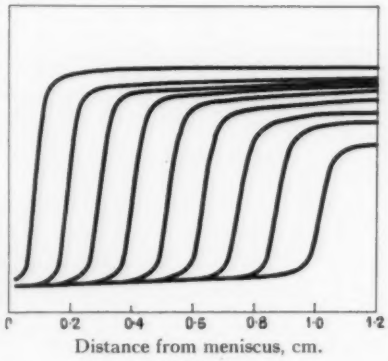


FIG. 25*b*.

FIG. 25. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Helix nemoralis* at pH 8.5 (s_{20} = 16.6); centrifugal force 125,000; time between exposures 5 minutes.

Some runs indicate that in more dilute solutions the amount of C increases. At pH 8.2 B and C are dissociated into a uniform component H with sedimentation constant 15.0. H is the single molecular species present in the pH-region 8.0–8.7. At pH 8.7 a small part of it is split up into a fourth component I with the sedimentation constant

11.1. H and I are present together in the entire pH-region investigated above pH 8.7. The amount of I increases with pH. At pH 2.9 and below the hemocyanin is inhomogeneous. Figure 23 gives an example of a sedimentation run and Fig. 24 (Plate III) the pH-stability curve.

Helix nemoralis

Upsala, Sweden

The main component, B, of this hemocyanin, has the sedimentation constant 101.0. It is present in the pH-range 3.6–8.0. Between pH 3.6 and 4.2 it is accompanied by component C with the sedimentation constant 65.0. At pH 7.7 and 8.0 there are also traces of C. Above pH 8 B and C are split up into the very homogeneous dissociation product H with the sedimentation constant 16.6. H is present alone up to 10.5 where I with the sedimentation constant 11.9 appears. H and I coexist to 10.8. In the region 10.8–11.5 I is alone. At 11.5 another dissociation product, still lower, is present together with I. Around pH 3 the material is rather inhomogeneous. At pH 2.5 H and I are formed. Figure 25 gives an example of a sedimentation run and Fig. 26 (Plate III) the pH-stability curve.

Helix hortensis

Upsala, Sweden

In the pH-region 4.0–8.0 this hemocyanin has two components, B with the sedimentation constant 100.0 and C with the sedimentation constant 61.9. B is the main component, C only forms 15 per cent of the whole protein up to pH 7.2. Between 7.2 and 8.0 B is present in a higher relative amount. In the pH-range 8–9.7 the material is split up into component H with the sedimentation constant 15.9. At 9.7 the splitting goes further, beside H component I with the sedimentation constant 12.2 is present. At pH 11.3 and above H disappears. In the region 3.6–4.0 C is present alone and in more acid solutions the hemocyanin is split up into inhomogeneous, more low-molecular dissociation products. Figure 27 (Plate III) gives the stability diagram.

Limax maximus

Värmdön, Sweden

The main component, B, has the sedimentation constant 97.3. It is present in the pH-range 4.0–8.0 together with a higher component, A. A is rather inhomogeneous and low in concentration, therefore the determination of its sedimentation constant is uncertain. As mean value 136 has been found. At pH 4.0–4.2 a lower component C with

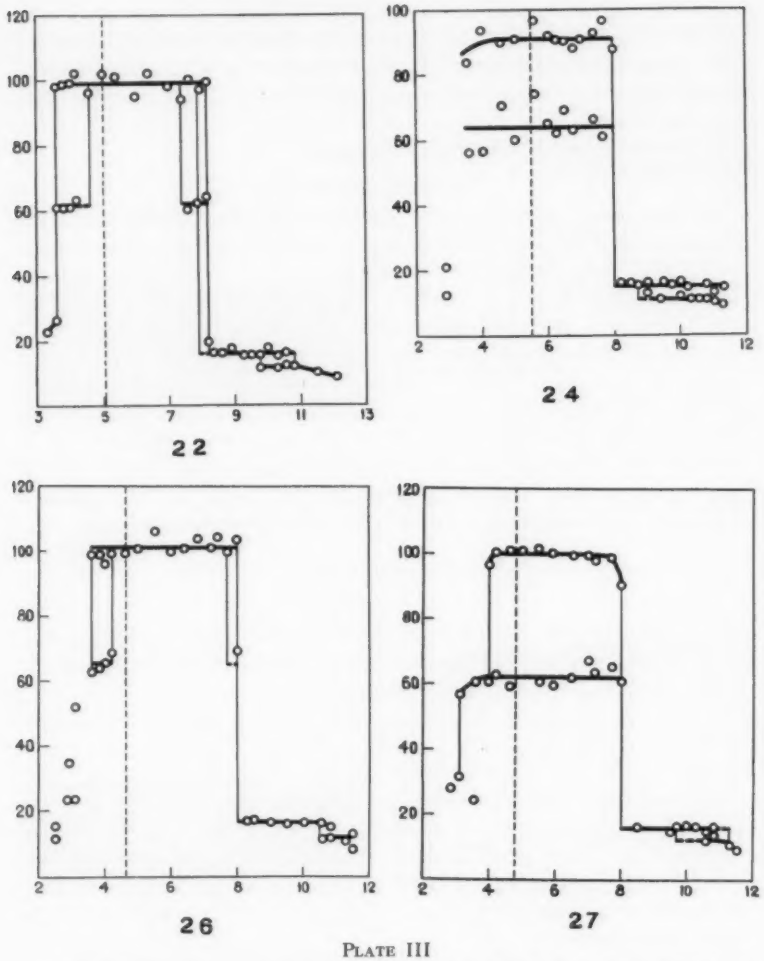


PLATE III

pH-stability diagrams for hemocyanins of species listed below. Abscissæ in all figures, pH; ordinates, s_{20}. The dotted lines indicate the positions of the isoelectric points.

FIG. 22. *Helix pomatia.*
FIG. 24. *Helix arbustorum.*
FIG. 26. *Helix nemoralis.*
FIG. 27. *Helix hortensis.*

the sedimentation constant 61.4 appears. At pH 3.6–3.8 the hemocyanin is further dissociated into three components, G, H, and I, with the sedimentation constants 25.5, 18.0, and 13.7. Above pH 8 A and B are dissociated into component H. Figure 28 (Plate IV) gives the pH-stability diagram.

TABLE XV

Helix nemoralis

Dilution of blood 10 times; centrifugal force in the pH-range 2–8 50,000 (speed 26,000 r.p.m.); above pH 8 210,000 (speed 54,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	s_{20} I	
HCl, Na-citrate, NaCl . . .	2.5	0.30			15.0	11.3	
" " " . . .	2.9	0.30					34.7, 23.2 inhomogeneous
" " " . . .	3.1	0.30					52.3, 23.7 inhomogeneous
HAc, NaAc, NaCl	3.6	0.20	98.8	62.7			
" " "	3.8	0.20	99.3	63.4			
" " "	4.0	0.20	96.9	65.5			
" " "	4.2	0.20	98.9	68.4			
" " "	4.3	0.20	99.5				
" " "	4.6	0.20	99.1				
KH_2PO_4, Na_2HPO_4, NaCl	5.0	0.20	100.4				
" " "	5.5	0.20	104.4				
" " "	5.6	0.20	107.7				
" " "	6.0	0.20	99.5				
" " "	6.4	0.20	100.7				
" " "	6.8	0.20	103.4				
" " "	7.2	0.20	101.0				
" " "	7.4	0.20	103.7				
" " "	7.7	0.20	100.1	+			
" " "	7.7	0.20	99.5	+			
" " KCl .	8.0	0.12	104.9	69.4			
" $Na_2B_4O_7$, NaCl .	8.3	0.12			16.5		
" " " .	8.5	0.25			16.8		
" " " .	9.0	0.25			16.6		
Na_2CO_3, $Na_2B_4O_7$, NaCl . .	9.5	0.25			16.2		
" " " . .	10.0	0.25			16.4		
" " " . .	10.5	0.25			16.9	+	
" " " . .	10.6	0.25			15.2	11.6	
" " " . .	10.8	0.25			15.1	11.8	
NaOH, Na_2HPO_4, NaCl . .	11.3	0.30				10.5	
" " " . .	11.5	0.30				12.7	9.0
" " " . .	11.5	0.30				12.8	8.5
			101.0	65.0	16.6	11.9	

Conchifera: Cephalopoda:

Decapoda. Hemocyanins from three animals belonging to this group: *Sepia officinalis*, *Rossia owenii*, and *Loligo vulgaris*, have been investigated. The main component of their blood has the sedimentation constants 55.9, 56.2, and 56.7 respectively. They are identical within limits of experimental error. Moreover, their final alkaline dissociation products are the same. *Sepia* and *Loligo* have components with the sedimentation constants 18.7 and 16.9. These probably represent a splitting of the original molecule into the same number of

TABLE XVI

Helix hortensis

All conditions as in Table XIV

Solvent	pH of solvent	Total molar	s_{20} B	s_{20} C	s_{20} H	s_{20} I	
Na-citrate, HCl, NaCl . . .	2.5	0.20					Very inhomogeneous
" " " . . .	2.9	0.30					28.1, 19.3, 13.0
							Very inhomogeneous
" " " . . .	3.1	0.30		56.7*			31.1 inhomogeneous
HAc, NaAc, NaCl	3.6	0.20		60.7			24.0 inhomogeneous
" " "	4.0	0.20	96.5	60.3			
" " "	4.2	0.20	100.5	62.8			
" " "	4.6	0.20	101.1	58.9			
" " "	5.0	0.20	100.9	+			
KH_2PO_4, Na_2HPO_4, NaCl	5.5	0.20	101.5	60.8			
" " "	5.9	0.20	100.6	59.3			
" " "	6.5	0.20	100.1	61.4			
" " "	6.6	0.20	98.6	62.0			
" " "	7.0	0.20	99.5	66.7			
" " "	7.2	0.20	98.0	63.4			
" " "	7.7	0.20	99.1	65.4			
" " KCl .	8.0	0.12	+	+			
" $Na_2B_4O_7$, NaCl . .	8.5	0.23			16.6		
Na_2CO_3, $Na_2B_4O_7$, NaCl . .	9.5	0.23			14.8		
" " " . .	9.7	0.20			16.5	+	
" " " . .	10.0	0.23			16.5	+	
" " " . .	10.3	0.23			16.3	+	
" " " . .	10.6	0.20			14.7	12.3	
" " " . .	10.8	0.23			15.8	13.8	
NaOH, Na_2HPO_4, NaCl . .	11.3	0.30			+	10.3	
" " " . .	11.5	0.30				8.99*	
			100.0	61.9	15.9	12.1	

* Not used for the calculation of mean value.

fragments. In all three of them there is observed an inhomogeneous component of rather high sedimentation constant which exists in a narrow range inside the pH-region where the main component is stable.

Octopoda. This group is represented by two animals, *Octopus vulgaris* and *Eledone moschata.* The normal sedimentation constants for the hemocyanin in their blood are 49.3 for *Octopus* and 49.1 for *Eledone.* Only one dissociation product is found and this is identical

TABLE XVII

Limax maximus

Dilution of blood 10 times; centrifugal force in the pH-range 3.6–8.0 50,000 (speed 26,000 r.p.m.); above 8.0 175,000 (speed 50,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	s_{20} A	s_{20} B	s_{20} C	s_{20} G	s_{20} H	s_{20} I
HAc, NaAc, NaCl	3.6	0.20				24.3	18.6	13.7
" " "	3.8	0.20				26.6	19.9	13.6
" " "	4.0	0.20	+	95.2	60.8			
" " "	4.2	0.20	143.0	96.3	63.0			
" " "	4.6	0.20	+	100.7				
" " "	5.0	0.20	126.3	97.0				
" " "	5.0	0.20	+	100.5				
KH_2PO_4, Na_2HPO_4, NaCl	5.6	0.20	+	97.8				
" " "	5.9	0.20	128.9	93.0				
" " "	6.4	0.20	129.1	93.2				
" " KCl	6.8	0.20	+	99.3				
" " NaCl	2.2	0.20	153.1	92.4				
" " "	7.4	0.20	+	96.1				
" " "	8.0	0.20	+	105.6				
" $Na_2B_4O_7$, NaCl	8.5	0.25					17.8	
" " "	9.0	0.25					17.1	
Na_2CO_3, $Na_2B_4O_7$, NaCl	10.0	0.25					16.6	
			136.0	97.3	61.9	25.5	18.0	13.7

with the final one in the blood from the decapods. The molecular weight of this small component probably represents 1/7 of the normal molecular weight of the octopod hemocyanin and 1/8 of the molecular weight of the decapod hemocyanin.

Loligo vulgaris

Naples, Italy

The hemocyanin appears in four different molecular species, namely, the components D, F, H, and I. Of those D, H, and I are well-defined and homogeneous; they give the sedimentation constants 56.7, 16.9, and 12.1. F is very inhomogeneous and it appears irregu-

larly. The mean value of its sedimentation constant is 35.9.

Component D exists in the pH-range 5.0–10.0 with the exception of a narrow region around 8. It is present alone between pH 5.0 and 7.2, accompanied by H from pH 7.2 to 10.0 and by F from 7.7 to 8.7. Component I appears together with D and H in the pH-range 9.6–10.0 and is the single component present above 10.0. Above pH 11 it is

TABLE XVIII

Loligo vulgaris

Dilution of blood 10 times; centrifugal force in the pH-range 5–10 60,000 (speed 30,000 r.p.m.); above pH 10 260,000 (speed 60,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	S_{20} D	S_{20} F	S_{20} H	S_{20} I
HAc, NaAc, NaCl	5.0	0.20	55.6			
KH_2PO_4, Na_2HPO_4, NaCl	5.5	0.20	55.9			
" " "	5.9	0.20	56.3			
" " "	6.5	0.20	56.9			
" " "	6.8	0.20	54.1			
" " "	7.2	0.20	58.9		21.2	
" " "	7.7	0.20	57.9	39.3		
" " "	8.0	0.20		+	18.9	
" " "	8.0	0.20		31.5	18.3	
" $Na_2B_4O_7$, "	8.4	0.25	57.8	+	+	
" " "	8.7	0.25	57.1	37.0	16.6	
" " "	9.2	0.25	57.5		14.0	
Na_2CO_3, " "	9.4	0.25	57.6		17.5	
" " "	9.6	0.25	55.3		16.9	12.6
" " "	9.8	0.25	+		16.2	12.5
" " "	10.0	0.25	56.6		+	+
" " "	10.3	0.25				12.0
" " "	10.6	0.25				11.4
" " "	11.1	0.27				10.5
" " "	11.5	0.30				9.4*
" " "	11.7	0.35				9.0*
			56.7	35.9	16.9	12.1

* Not used for the calculation of mean value.

still perfectly homogeneous with regard to molecular weight but its sedimentation constant drops gradually. This must be due to a change in shape of the particles. Below pH 5 aggregation sets in. Figures 29 and 30 give examples of sedimentation runs, Fig. 31 (Plate IV) gives the pH-stability curve.

Rossia owenii

Kristineberg, Sweden

The main component, D, has the sedimentation constant 56.2. It

exists between pH 5.0 and 10.1; it is present alone in the pH-ranges 5.0–7.7 and 8.9–9.4, and together with component F in the pH-range 7.7–8.9, and together with component I in the pH-range 9.4–10.1. Component F is very inhomogeneous and present in small quantity, therefore its sedimentation constant is not well-defined. In one run the value 40.9 was found. Component I appears first in small quantity

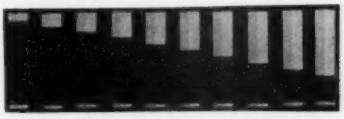


FIG. 29*a*.

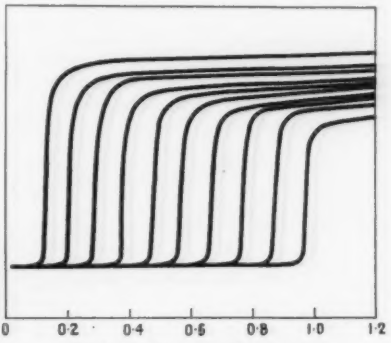


FIG. 29*b*.

FIG. 29. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Loligo vulgaris* at pH 6.8 (s_{20} = 56.7); centrifugal force 60,000; time between exposures 5 minutes.

FIG. 30*a*.

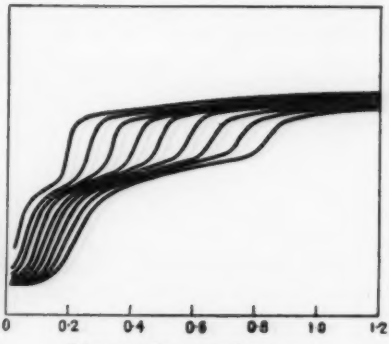


FIG. 30*b*.

FIG. 30. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Loligo vulgaris* at pH 7.7 (s_{20} = 56.7 and 35.9); centrifugal force 50,000; time between exposures 5 minutes.

together with D, but its relative amount increases with pH. Above pH 10.1 it is present alone. It shows a behavior similar to component I in the *Loligo*-blood. In solutions more acid than pH 5 the hemocyanin is precipitated. Figures 32 and 33 give samples of sedimentation runs and Fig. 34 (Plate IV) the pH-stability curve.

TABLE XIX

Rossia owenii

Dilution of blood 15 times; centrifugal force below pH 10 60,000 (speed 30,000 r.p.m.); above pH 10 215,000 (speed 55,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	S_{20} D	S_{20} F	S_{20} I
HAc, NaAc, NaCl	5.0	0.24	59.3		
KH_2PO_4, Na_2HPO_4, NaCl	5.5	0.24	56.0		
" " "	5.9	0.24	55.0		
" " "	6	0.24	53.7		
" " "	6.4	0.24	52.9		
" " "	6.8	0.24	57.0		
" " "	7.4	0.24	53.8		
" " "	7.7	0.24	57.7	+	
" $Na_2B_4O_7$, "	8.4	0.30	56.4	+	
" " "	8.9	0.30	57.5	40.9	
Na_2CO_3, " "	9.2	0.30	56.5		
" " "	9.4	0.30	56.3		+
" " "	9.8	0.30	58.8		+
" " "	10.1	0.30	59.0		13.0
" " "	10.3	0.30			11.4
" " "	10.6	0.30			10.4
NaOH, Na_2HPO_4, NaCl	11.1	0.39			10.3
" " "	11.5	0.47			10.4
" " "	11.7	0.40			10.0
			56.2		10.9

Sepia officinalis

Roscoff, France

The sedimentation picture of this blood shows two components, D and H, in the pH-range 4.0–8.5. D has the sedimentation constant 55.9 whereas the sedimentation constant of H is 18.7. From pH 4 to 7 the proportions between D and G seem to be constant. From the centrifuge curves it can be estimated roughly that the percentage of D is 75, of H 25, assuming that they have the same light absorption. The amount of H rises towards the alkaline side. In the region 8.5–10.4 another component is observed, which is very inhomogeneous

and with a sedimentation constant higher than H. In this region the inhomogeneity of the material makes the measurements rather uncertain. At pH 10.6 a homogeneous dissociation product, I, is formed. Its sedimentation constant is originally 11.8 but drops gradually as in the case of *Loligo* and *Rossia*. At pH 4 an increase in the sedimentation constants of D and H shows that aggregation sets in. At 3.6 the

FIG. 32*a*.

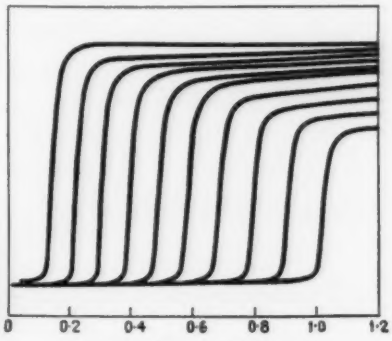


FIG. 32*b*.

FIG. 32. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Rossia owenii* at pH 6.0 (s_{20} = 56.2); (centrifugal force 70,000; time between exposures 5 minutes.

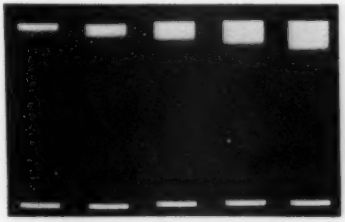


FIG. 33*a*.

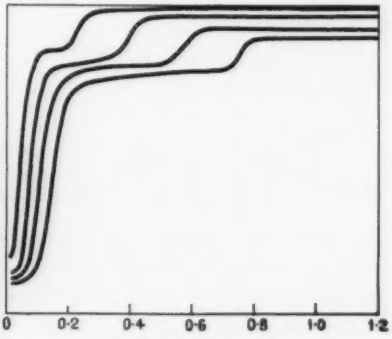


FIG. 33*b*.

FIG. 33. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Rossia owenii* at pH 10.1 (s_{20} = 56.2 and 13.0); centrifugal force 120,000; time between exposures 5 minutes.

protein is precipitated. Figures 35 and 36 give examples of sedimentation runs, Fig. 37 (Plate IV) the pH-stability diagram.

Octopus vulgaris

Roscoff, France

The main component, E, of this hemocyanin has the sedimentation constant 49.3. It is present in the blood in the pH-region 4.0–10.3.

TABLE XX

Sepia officinalis

All conditions the same as in Table XVIII.

Solvent	pH of solvent	Total molar	S_{20} D	S_{20} H	S_{20} I	
NaAc, HAc, NaCl	4.0	0.20	62.6*	27.3*		Inhomogeneous
" " "	4.6	0.20	58.9	23.9		
" " "	5.0	0.20	53.4	18.9		
" " "	5.5	0.20	56.8	15.2		
KH_2PO_4, Na_2HPO_4, NaCl	6.0	0.20	54.7	20.9		
" " "	6.5	0.20	53.5	18.3		
" " "	6.8	0.20	57.1	17.6		
" " "	6.8	0.20	57.2	+		
" " "	7.4	0.20	55.4	18.3		
" " "	8.0	0.20	49.8	18.9		
" $Na_2B_4O_7$, "	8.5	0.25	54.3	18.5†		
Na_2CO_3, " "	9.0	0.25	55.2	+†		
" " "	9.4	0.25	58.0	20.0*†		
" " "	10.0	0.25	+	20.5*		Inhomogeneous
" " "	10.6	0.25			11.8	
NaOH, Na_3HPO_4, NaCl	11.1	0.30			10.5	
" " "	11.5	0.30			10.9	
" " "	11.7	0.30			9.1	
			55.9	18.7	10.6	

* Not used for the calculation of mean value.

† Three components present, the third with a sedimentation constant between D and H. At 9.4 the value 42 was obtained.

At pH 9.5 it is dissociated to a small degree into component I. The relative amount of I increases with pH. It appears together with E in the pH-region 9.5–10.3, but above pH 10.3 it is alone. The sedimentation constant at pH 10.3 is 12.1, but above it drops. I seems to be identical with the alkaline dissociation products of the decapods. In the pH region 4.0–9.5 E is generally the only component present; in three runs it was accompanied by a lower component each time with different sedimentation constant. Figure 38 (Plate IV) gives the pH-stability diagram.

The pH-stability diagram has been studied here before (Svedberg and Eriksson, 1932). If the value given there for the sedimentation constant of the main component is corrected for the influence of density and viscosity of the buffer solutions, it becomes 52.6. This agrees fairly well with the present determination. After correction

FIG. 35*a*.

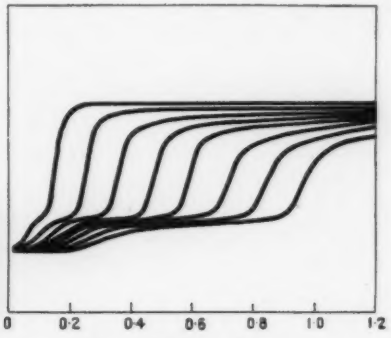


FIG. 35*b*.

FIG. 35. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Sepia officinalis* at pH 5.5 (s_{20} = 55.4 and 18.7); centrifugal force 71,000; time between exposures 5 minutes.

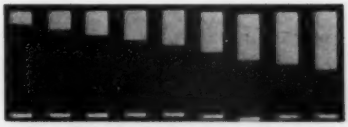


FIG. 36*a*.

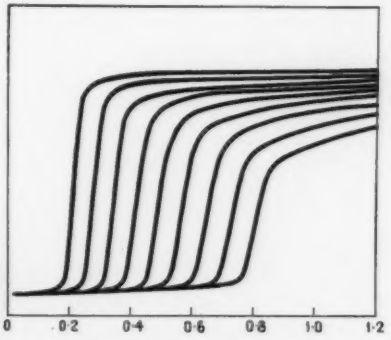


FIG. 36*b*.

FIG. 36. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Sepia officinalis* at pH 11.5 (s_{20} = 10.6); centrifugal force 220,000; time between exposures 5 minutes.

this value is constant inside the pH-region in which the main component is present. During the earlier investigation the blood was not stored in frozen condition as now and was dialyzed for some days against the buffer instead of being diluted just before the run. This might explain the appearance of a low-molecular component with a sedimentation constant changing with pH. A similar component has only appeared in a few runs in this work.

TABLE XXI

Octopus vulgaris

Dilution of blood 20 times; centrifugal force 100,000 (speed 37,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	S_{20} E	S_{20} I	S_{20}
NaAc, HAc, NaCl	4.0	0.20	51.6		
" " "	4.6	0.20	52.4		
" " "	5.0	0.20	49.9		
Na_2HPO_4, KH_2PO_4, NaCl	5.6	0.20	47.6		
" " "	6.0	0.20	48.8		
" " "	6.3	0.20	48.5		13.7
" " "	6.8	0.20	47.6		
" " "	7.3	0.20	48.0		
" " "	7.7	0.20	48.9		20.1
" " "	8.0	0.20	47.8		15.2
$Na_2B_4O_7$, " "	8.4	0.25	45.7		
" " "	8.8	0.25	45.2		
" Na_2CO_3, "	9.0	0.25	49.5		
" " "	9.3	0.25	51.1		
" " "	9.5	0.25	51.9	+	
" " "	9.8	0.25	52.8	+	
" " "	10.3	0.25	51.4	12.1	
" " "	10.8	0.25		11.3	
			49.3		

Eledone moschata

Naples, Italy

The main component E of the hemocyanin is present from pH 5 to 11.0. The sedimentation constant is 49.1. At 10.6 about ten per cent of the protein splits up into smaller molecules having the sedimentation constant of 11.8 (component I). At 11.0 the percentage of the lighter protein goes up to 50. At pH 11.1 the splitting is complete. The sedimentation constant of the very uniform dissociation product drops with increasing pH. At pH 5.4 the protein is very inhomogeneous. In more acid solutions precipitation sets in.

Figures 39 and 40 give examples of sedimentation runs, Fig. 41 (Plate IV) the pH-stability diagram.

Reversibility

Some experiments have been carried out on the reversibility of the dissociation reactions and the equilibrium between the dissociation

Table XXII

Eledone moschata

Dilution of blood 15 times; centrifugal field 75,000 (speed 32,000); in the four last runs 230,000 (speed 57,000 r.p.m.); thickness of column of solution 1.2 cm.; other conditions as in Table II.

Solvent	pH of solvent	Total molar	S_{20} E	S_{20} I	
HAc, NaAc, NaCl.....	5.4	0.20	49.4		Very inhomogeneous
KH_2PO_4, Na_2HPO_4, NaCl.....	5.9	0.20	49.2		
" " "	6.2	0.20	47.8		
" " "	6.5	0.20	48.7		
" " "	6.8	0.20	48.1		
" " "	7.1	0.20	46.7		
" " "	7.4	0.20	48.5		
" " "	7.7	0.20	49.7		
" " "	8.0	0.20	50.0		
" $Na_2B_4O_7$, "	8.5	0.25	48.2		
Na_2CO_3, " "	9.1	0.25	50.8		
" " "	9.5	0.25	50.3		
" " "	9.8	0.25	49.9		
" " "	10.3	0.25	50.1		
" " "	10.6	0.25	49.8	11.8	
" " "	11.0	0.25	48.2	10.5	
" " "	11.1	0.25		11.4	
NaOH, Na_2HPO_4, NaCl.....	11.5	0.30		9.46	
" " "	11.5	0.30		9.59	
" " "	11.7	0.30		8.57	
			49.1	10.60	

products. Two hemocyanins have been chosen for these tests, viz. the blood pigments of *Helix pomatia* and *Limulus polyphemus*. They can be regarded as representatives for two different groups. In the natural blood and in a large part of the investigated pH-region the *Helix* hemocyanin consists of just one molecular species. The *Limulus* hemocyanin, on the contrary, contains four different components in its natural state and inside the main part of the stability region (from pH 5 to 10.5).

Helix pomatia

The reversibility of the dissociation caused by a change in pH was studied. The results are summarized in Table XXIII. The blood was taken to pH_I, chosen so that some sort of dissociation took place, and then to pH_{II}; where it normally would have been less dissociated. In Table XXIII are given the values for the sedimentation constants

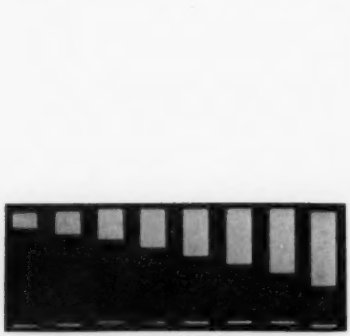


FIG. 39*a*.

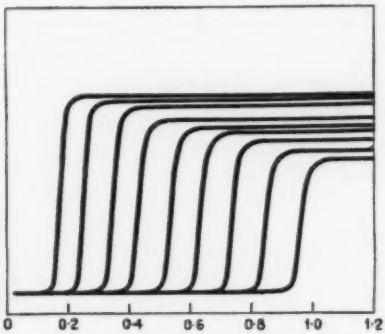


FIG. 39*b*.

FIG. 39. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Eledone moschata* at pH 9.8 (s_{20} = 49.1); centrifugal force 72,000; time between exposures 5 minutes.

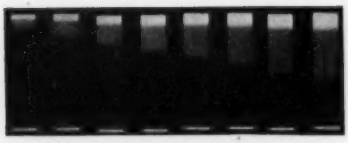


FIG. 40*a*.

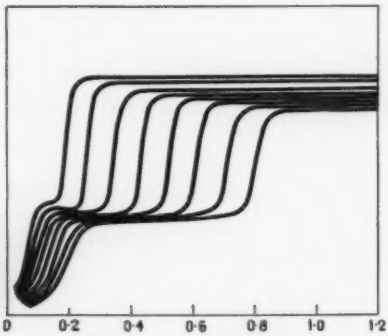


FIG. 40*b*.

FIG. 40. Sedimentation pictures (*a*) and photometric records (*b*) for hemocyanin from *Eledone moschata* at pH 11.0 (s_{20} = 49.1 and 10.6); centrifugal force 72,000; time between exposures 5 minutes.

at pH_I, the normal sedimentation constants at pH_{II}, and the sedimentation constants found at pH_{II} when the protein had first been taken to pH_I. The results show that the dissociation is reversible inside a certain pH-region, but if the solution is made too alkaline only a part of the hemocyanin is associated into the normal components. Another part of it aggregates, but into smaller particles than normally found. If the solution is brought to pH 12 only inhomogeneous material is formed by neutralization.

The equilibrium between the components was studied in the

TABLE XXIII

Helix pomatia

pH_I	pH_{II}	s_{20}	At pH_I		s_{20} for comp. normal at pH_{II}		s_{20} for comp. found at pH_{II}			
8.0	6.8	98.9	62.0	16.0	98.9		95.7			
8.9	6.8			16.0	98.9		99.0			
8.6	7.7			16.0	98.9	62.0		60.7	17.9	Inhomogeneous
9.5	5.9		16.0	12.1	98.9		106.8	66.1	22.3	
10.3	5.0		16.0	12.1	98.9		94.1	63.4	23.0	
10.5	6.8		16.0	12.1	98.9		97.9	64.8	26.2	
10.8	5.9		16.0	12.1	98.9		92.8	60.1	22.7	
10.8	7.7		16.0	12.1	98.9	62.0	+	59.8	+	
10.8	5.9		16.0	12.1	98.9		109.8	70.5	23.6	
12.1	5.9		Inhomogeneous		98.9			32.3		Inhomogeneous
4.0	6.8	98.9	62.0		98.9		85.7			Two components, bad separation
4.0	5.4	98.9	62.0		98.9		100.9			

following way: a solution at pH 8.0, containing the components B and C, was run in the ultracentrifuge until component B had sedimented to the bottom of the cell. The centrifuge was stopped and the liquid pipetted off. The sediment was stirred up in a small quantity of the same buffer. Runs were made on these two solutions. The initial solution contained 67 per cent B and 33 per cent C, the solution pipetted off 73 per cent C and 27 per cent B and the sediment 67 per cent C and 33 per cent B. These figures agree within limits of error. The relative amounts are obtained from the microphotometric curves of the sedimentation pictures assuming that the different components have the same light absorption. The dissociation products seem to be in equilibrium. If the relative concentration of one is diminished the same proportions are reëstablished.

Limulus polyphemus

The reversibility on changing the pH was studied at pH 10.8, where the protein is dissociated into a product with sedimentation constant 5.87, and at 4.0, where it contains three components. The dissociation reactions were completely reversible.

In order to study the equilibrium between the components the same procedure as in the case of the *Helix* hemocyanin was carried out. A solution containing the four components D, F, H, and K was centrifuged until D had reached the bottom. In the initial solution the composition was:

22 per cent D, 14 per cent F, 41 per cent H, and 23 per cent K.

In the pipetted-off solution

14 per cent D, 11 per cent F, 55 per cent H, and 20 per cent K.

In the sediment

58 per cent D, 17 per cent F, 25 per cent of H and K together.

Evidently the sediment contains a larger percentage of the heavy component D and less of the light components H and K, while the supernatant solution contains less of D and more of H and K.

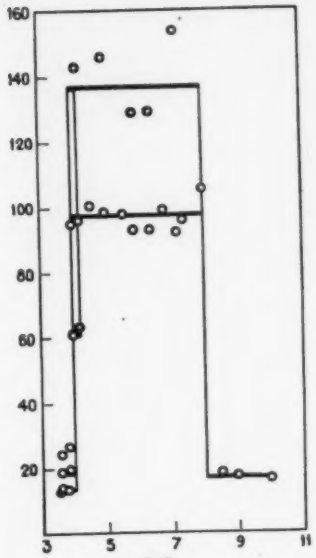
The solutions were stored for a week and run again. The values then obtained agreed with those given above within the limits of error. The components therefore do not seem to be in equilibrium in this case.

If the components are in equilibrium the equilibrium must be disturbed during the ordinary runs. The concentration of the higher components in the solution decreases faster than the concentration of the lower components. Whether or not this shows in the sedimentation diagrams must depend on the rate of reëstablishment of the equilibrium and the time taken by the run. In some cases such an effect has been observed.

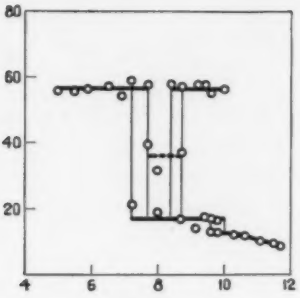
EXPLANATION OF PLATE IV

pH-stability diagrams for hemocyanins of species listed below. Abscissæ in all figures, pH; ordinates, s_{20}.

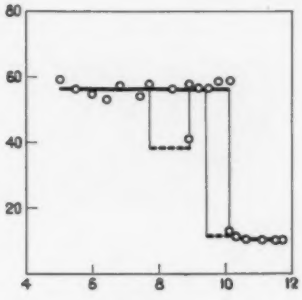
FIG. 28. *Limax maximus.*
FIG. 31. *Loligo vulgaris.*
FIG. 34. *Rossia owenii.*
FIG. 37. *Sepia officinalis.*
FIG. 38. *Octopus vulgaris.*
FIG. 41. *Eledone moschata.* The dotted line in Fig. 41 indicates the position of the isoelectric point.



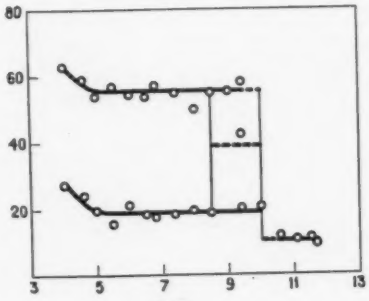
28



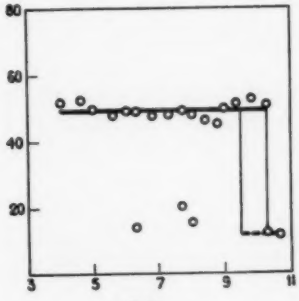
31



34



37



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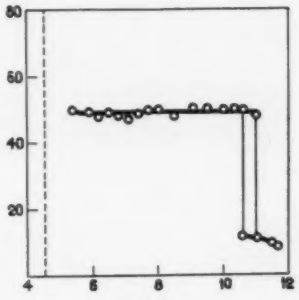

41

PLATE IV

Molecular Weights

A number of sedimentation equilibrium measurements have been carried out so as to make it possible to coördinate sedimentation constants and molecular weights. In cases where more than one molecular species is present the computation of molecular weights from equilibrium data is uncertain and we have therefore refrained

Fig. 42*a*.

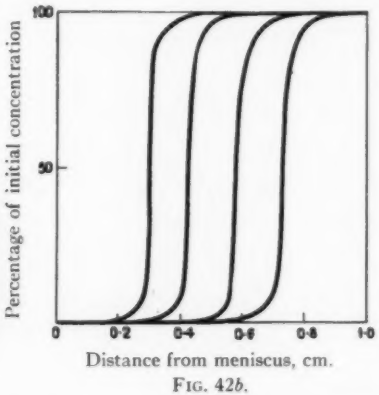

Fig. 42*b*.

Fig. 42. Sedimentation pictures (*a*) and concentration curves (*b*) for hemocyanin from *Helix pomatia* at pH 5.5 (s_{20} = 98.9); centrifugal force 45,000; time between exposures 5 minutes.

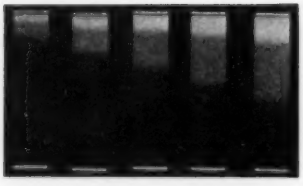

Fig. 43*a*.

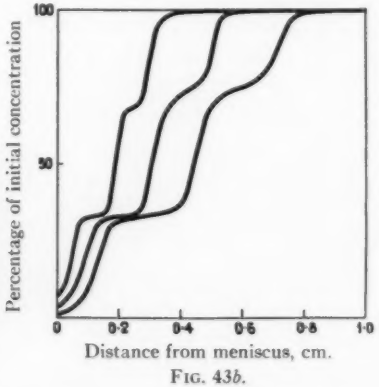

Fig. 43*b*.

Fig. 43. Sedimentation pictures (*a*) and concentration curves (*b*) for hemocyanin from *Helix pomatia* at pH 8.2 (s_{20} = 98.9, 62.0, and 16.0); centrifugal force 60,000; time between exposures 5 minutes.

from studying solutions of this kind. The results are summarized in Table XXIV.

Discussion of Results

The most striking result of this investigation is perhaps the perfect homogeneity of the various hemocyanins with regard to molecular weight. Not only the main component or components which exist in the region comprising the isoelectric point (Fig. 42) but also the

Table XXIV

Sedimentation equilibrium determinations on hemocyanins

Species	Dilution of blood	Solvent	Total molar	pH	Thickness of cell	r.p.m.	Method	Time in hours	M	M Mean
Pandalus borealis	7	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	4	3800	Scale	160	408000	397000
	7	" " "	0.20	6.8	4	3800	Scale	132	386000	
Palinurus vulgaris	10	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	12	3330	Slit	120	485000	447000
	10	" " "	0.20	6.8	12	3060	Scale	112	462000	
	10	" " "	0.20	6.8	12	3540	Scale	154	450000	
	10	" " "	0.20	6.8	12	3300	Scale	131	409000	
	10	" " "	0.20	6.8	12	3300	Scale	117	429000	
Homarus vulgaris	7	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	12	3700	Slit	137	793000	803000
	7	" " "	0.20	6.8	12	3700	Slit	112	798000	
	7	" " "	0.20	6.8	4	3700	Scale	214	817000	
Calocaris macandræ	4	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	4	2600	Scale	249	1.306000	1.329000
	4	" " "	0.20	6.8	4	2700	Scale	226	1.352000	
Helix pomatia	20	Na_2HPO_4, KH_2PO_4, NaCl	0.20	6.8	12	1500	Slit	168	6.520000	6.680000
	60	" " "	0.20	6.8	12	1500	Slit	208	6.658000	
	120	" " "	0.20	6.8	12	1620	Scale	156	6.864000	
	20	KH_2PO_4, $Na_2B_4O_7$, NaCl	0.25	8.5	4	3180	Slit	161	819000	797000
	20	" " "	0.25	8.5	4	3162	Scale	238	774000	

products of reversible dissociation (Fig. 43) are definite molecular individuals. At very low and very high pH-value where irreversible dissociation occurs the products are inhomogeneous (Fig. 44). Compare with Fig. 45.

The components of highest molecular weight always exist in a region comprising the isoelectric point. As a rule the number of components increase from the isoelectric point towards the alkaline and acid side. Among the 22 species studied 13 have only one component at the isoelectric point, 7 have two, 1 three and 1 four components.

The sedimentation constants observed may be arranged in ten classes (A–K), as shown by Table XXV. For each class the variations are almost within the limit of experimental error. To a certain molecular weight may correspond more than one sedimentation

constant according to the more or less pronounced deviation from the spherical shape which the molecule shows (Table XXVI). The molecular weights of the hemocyanin found in the blood of a certain species are always simple multiples of the lowest well-defined compo-

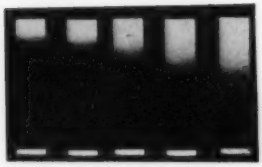

FIG. 44*a*.

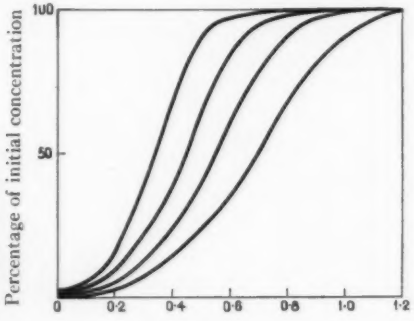

FIG. 44*b*.

FIG. 44. Sedimentation pictures (*a*) and concentration curves (*b*) for hemocyanin from *Nephrops norvegicus* at pH 11.7 (s_{20} = 8.95); centrifugal force 220,000; time between exposures 10 minutes.

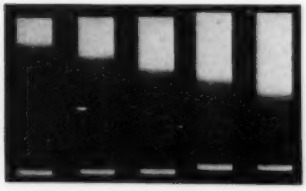

FIG. 45*a*.

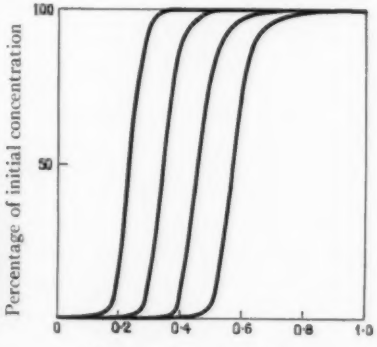

FIG. 45*b*.

FIG. 45. Sedimentation pictures (*a*) and concentration curves (*b*) for hemocyanin from *Nephrops norvegicus* at pH 4.5 (s_{20} = 24.5); centrifugal force 150,000; time between exposures 5 minutes.

nent. Thus for the Malacostraca the relationship is 1 : 2 and for the Gastropoda 2 : 8 : 16 : 24. Moreover, the weights of all the well-defined hemocyanin molecules seem to be simple multiples of the lowest one among them (Table XXVI).

In most cases the hemocyanin components in the blood of a certain species are interconnected by reversible, pH-influenced dissociation-

TABLE XXV

Sedimentation constants arranged in classes

Species	A	B	C	D	E	F	G	H	I	K
CRUSTACEA:										
Malacostraca										
Pandalus borealis							22.9	17.4		+
Palinurus vulgaris								16.4		4.10
Nephrops norvegicus							24.5	17.1		5.97
Homarus vulgaris							22.6	16.1		+
Astacus fluviatilis							23.3	16.3		4.02
Carcinus mænas							23.3	16.7		+
Cancer pagurus						32.7	23.6	16.4		4.71
ARACHNOMORPHA:										
Xiphosura										
Limulus polyphemus				56.6		34.6	24.0	16.1		5.87
CONCHIFERA:										
Gastropoda										
Littorina littorea		99.7	63.3					15.0		
Neptunea antiqua		104.0						14.3	+	
Buccinum undatum		102.1	63.8						+	
Busycon canaliculatum	130.4	101.7	61.1					13.5		
Helix pomatia		98.9	62.0					16.0	12.1	
" *arbustorum*		91.2	64.1					15.0	11.1	
" *nemoralis*		101.0	65.0					16.6	11.9	
" *hortensis*		100.0	61.9					15.9	12.1	
Limax maximus	136.1	97.3	61.9				25.5	18.0	13.7	
Cephalopoda										
Decapoda										
Loligo vulgaris				56.7		35.9		16.9	12.1	
Sepia officinalis				55.9		+		18.7	10.6	
Rossia owenii				56.2		+			10.9	
Octopoda										
Octopus vulgaris					49.3				+	
Eledone moschata					49.1				10.6	

association reactions. At certain critical pH values a profound change in the number and percentage of the components takes place. The shift in pH necessary to bring about the reaction is not more than a few tenths of a unit. Consequently the forces holding the dissociable parts of the molecule together must be very feeble. This fact makes it all the more surprising that an equal backward shift in pH does

really cause an association of the parts to exactly the same molecule. For this reason it is justified, we think, to use the term molecule for the hemocyanin particles despite their enormous size. Their mass cannot be changed continually as in the case of an ordinary colloid particle and they have a finite stability range with regard to the surroundings. The hemocyanin particles, therefore, most probably possess a definite structure.

TABLE XXVI

Species	pH	S_{20}	D_{20}	M_s	M_e	$M_{calc.}$
Pandalus borealis	6.8	17.4			397,000	
Palinurus vulgaris	6.8	16.4	3.4	446,000	447,000	
Helix pomatia	9.7	12.1	2.23	502,000		1×406,000
Busycon canaliculatum	9.6	13.5	3.29	379,600		=12×33,800
Eledone moschata	11.6	10.6	2.16	457,000		
Nephrops norvegicus	6.8	24.5	2.79	812,000		
Homarus vulgaris	6.8	22.6	2.78	752,000	803,000	2×406,000
Helix pomatia	8.6	16.0	2.06	719,000	797,000	=812,000
Helix nemoralis	8.6	16.6	1.92	799,000		
Calocaris macandræ	6.8	34.0			1,329,000	4×406,000 =1,624,000
Octopus vulgaris	6.8	49.3	1.65	2,785,000		7×406,000
Eledone moschata	6.8	49.1	1.64	2,791,000		=2,842,000
Rossia owenii	6.8	56.2	1.58	3,316,000		8×406,000 =3,248,000
Helix pomatia	6.8	98.9	1.38	6,630,000	6,680,000	16×406,000
Busycon canaliculatum	6.8	101.7	1.38	6,814,000		=6,496,000
Busycon canaliculatum	6.8	130.4	1.17	9,980,000		24×406,000 =9,744,000

The pH-stability diagrams show a number of regularities indicative of biological kinship. All the Malacostraca give similar diagrams and so do the Gastropoda. The Cephalopoda show two types of diagram, one characteristic of the Decapoda and one of the Octopoda. Only one species of the Xiphosura, viz. the *Limulus*, has been studied. It shows a diagram entirely different from that of the Malacostraca species studied. The placing of *Limulus* among the crustaceans as was formerly done is therefore not supported by our sedimentation analysis of its blood pigment.

Within the large groups mentioned above an attempt at classification based upon the similarity of the stability diagrams would give, roughly, the following result:

Malacostraca: *Pandalus* and *Palinurus*—main comp. s = 16.
Nephrops and *Homarus*—main comp. s = 23.
Astacus, *Cancer* and *Carcinus*—both comp. s = 16 and s = 23 through most of the stability range.

Xiphosura: *Limulus*—four comp. s = 57, 34, 16, and 6 through most of the stability range; at the acid side also comp. s = 23.

Gastropoda: Pulmonata:
Helix and *Limax*—comp. s = 100 and 61 on both the alkaline and acid side of the isoelectric point.

Aspidobranchia:
Littorina, *Neptunea*, *Buccinum*, and *Busycon*—comp. s = 61 only on the alkaline side.

Cephalopoda, Decapoda:
Loligo, *Rossia*, and *Sepia*—main comp. s = 57.

Cephalopoda, Octopoda:
Octopus and *Eledone*—main comp. s = 49.

Really striking differences in the stability diagrams only occur for species belonging to different orders. All species of one and the same order have similar diagrams. Within the order Cephalopoda the two sections Decapoda and Octopoda show rather different diagrams, though. From the point of view of the blood sedimentation analysis, therefore, the Decapoda and the Octopoda ought perhaps to be regarded as systematic units of the same magnitude as the Gastropoda, i.e. as different orders within the class Mollusca, having developed as parallel branches from common ancestors far down in the Mollusca cladus. It is interesting to see that there is a notable difference between the diagrams for the species belonging respectively to the suborders Pulmonata and Aspidobranchia.

Similarities between species belonging to the same family or the same genus are sometimes revealed by their stability diagram (e.g. *Nephrops* and *Homarus; Helix pomatia* and *Helix nemoralis*) but species from two different although closely related genera may have very similar diagrams (e.g. *Helix hortensis* and *Limax maximus*).

Among the species studied by us no two have quite identical stability diagrams. It should be possible, therefore, to make a species determination based solely on the stability diagram. In such cases where the stability diagrams are rather similar (e.g. *Helix pomatia* and *Helix nemoralis*) a determination of the isoelectric point would settle the question (5.05 for *Helix pomatia* and 4.63 for *Helix nemoralis*) (Pedersen, 1933).

The expenses of this investigation have been defrayed by grants from the Andersson Foundation, the Astra Co., the Nobel Foundation, the Rockefeller Foundation and the Wallenberg Foundation. Living material of the following species were put at our disposal by the Zoölogical Station at Kristineberg, Sweden, viz. *Pandalus borealis*, *Nephrops norvegicus*, *Carcinus mænas*, *Buccinum undatum*, *Littorina littorea*, *Neptunea antiqua*, and *Rossia owenii*. For this valuable help we are indebted to Professor E. Lönnberg and Dr. G. Gustafson. Samples of blood from *Loligo vulgaris* and *Eledone moschata* were kindly sent us by Professor R. Dohrn and Dr. L. Califano of the Zoölogical Station at Naples. The *Limulus* and *Busycon* blood was given by Dr. A. C. Redfield of the Oceanographic Institution at Woods Hole, Massachusetts. Blood from *Palinurus vulgaris*, *Sepia officinalis* and *Octopus vulgaris* was collected for us at the Marine Station, Roscoff, France, by Dr. Lindahl. For the permission to make this collection we are indebted to Professor Charles Pérez.

Summary

1. Blood from twenty-two species containing hemocyanin as respiratory pigment has been subjected to a detailed ultracentrifugal study.

2. Sedimentation constants have been determined throughout the pH-stability ranges.

3. In some cases sedimentation equilibrium measurements have been carried out. From these data molecular weight values were obtained and correlated with the sedimentation constants.

4. The various hemocyanins are perfectly homogeneous with regard to molecular weight both at the isoelectric point and in the regions where reversible dissociation or association occurs.

5. The stability range for the components of highest molecular weight always comprises the isoelectric point.

6. Except at extremely low and high pH values the dissociation reactions are reversible.

7. The molecular weights of the different hemocyanins and their reversible dissociation products show a relationship of simple multiples.

8. Species belonging to the same order have similar pH-stability diagrams. Each species has a characteristic diagram by means of which it can be identified. In doubtful cases the isoelectric point may be used as complementary evidence.

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INDEX

ABRAMOWITZ, A. A. Physiology of the melanophore system in the catfish, Ameiurus, 259.

Abstracts of scientific papers presented at Marine Biological Laboratory, summer of 1936, 386.

Aenoplex smithii, spermatogenesis of, cytological phenomena, 375.

Alcohol, effect on gastrulation in Arbacia, 413.

Ameiurus, physiology of melanophore system, 259.

Arbacia punctulata, cleavage without nuclei, 101.

ARMSTRONG, P. B. Mechanism of hatching in Fundulus heteroclitus (abstract), 407.

Ascidians, excretion in, rôle of blood cells, 249.

BACTERIA, multiplication in different volumes stored sea water, influence of oxygen tension and solid surfaces, 324.

Bacteroids, intracellular, of roaches, 433.

BALLARD, W. W. Observations on lens regeneration in Amblystoma (abstract), 388.

BEAMS, H. W., AND R. L. KING. The effect of ultracentrifuging upon chick embryonic cells, with special reference to the "resting" nucleus and the mitotic spindle, 188.

BEAR, R. S. *See* Schmitt, Bear and Young (abstract), 402.

BISSONNETTE, T. H. Modified sexual photoperiodicity in ferrets, raccoons and quail (abstract), 395.

Blood cells, in excretion, ascidians, 249.

BOWEN, R. E. Sensory hairs of the dogfish ear, 429.

BOZLER, E. The double refraction of smooth muscle (abstract), 402.

BRAMBEL, C. E. *See* Cowles and Brambel, 286.

BROOKS, MATILDA M. The effect of methylene blue on the spectrophotometric picture of hemoglobin, CO-hemoglobin and CN-hemoglobin (abstract), 386.

BURTON, ALAN C. The basis of the principle of the master reaction in biology (abstract), 393.

——, —— —. *See* Sichel and Burton (abstract), 397.

CALCIUM, free, in action of stimulating agents on Elodea cells, 306.

CAMERON, J. A., AND K. O. MILLS. Behavior of frog tadpole epidermal cells during seven successive regeneration periods (abstract), 405.

CAMERON, J. A. *See* Fisher and Cameron (abstract), 404.

Carotenoid metabolism, in surf perches, structure and function of gut in, 217.

CAROTHERS, E. ELEANOR. Cellular behavior in abnormal growths produced by irradiation of grasshopper embryos (abstract), 394.

——, —. ——. Components of the mitotic spindle, 469.

Catfish, melanophore system, physiology of, 259.

CHAMBERS, R. Experimental studies on the oil wetting property of the plasma membrane (abstract), 398.

Chick embryonic cells, effect of ultracentrifuging, with reference to "resting" nucleus and mitotic spindle, 188.

Chromatophores, blue, in some freshwater Protozoa, 492.

Chromosomes, salivary gland, structure as affected by mechanical distortion, 238.

CLARK, E. R., AND ELEANOR L. CLARK. Observations on conditions affecting growth of cells and tissues, from microscopic studies on the living animal (abstract), 405.

CLARK, JEAN M. *See* Mazia and Clark, 306.

CLAUS, PEARL E. *See* Guyer and Claus, 462.

Cleavage without nuclei in Arbacia punctulata, 101.
CLOWES, G. H. A. *See* Keltch, Clowes and Krahl (abstract), 399.
——, —. —. —. *See* Krahl, Clowes and Taylor (abstract), 400.
COATES, C. W. *See* Smith and Coates, 282.
COE, W. R. Environment and sex in the oviparous oyster, Ostrea virginica, 353.
——, —. —. Sequence of functional sexual phases in Teredo, 122.
COONFIELD, B. R. Regeneration in Mnemiopsis leidyi Agassiz, 421.
COWLES, R. P., AND C. E. BRAMBEL. A study of the environmental conditions in a bog pond with special reference to the diurnal vertical distribution of Gonyostomum semen, 286.

DAN, KATSUMA, T. YANAGITA AND M. SUGIYAMA. The behavior of the cell surface during cleavage (abstract), 395.
DELAMATER, ARLENE JOHNSON. The effects of heavy water upon the fission rate and the life cycle of the ciliate, Uroleptus mobilis, 199.
Differentiation, energetics of, 59, 82.
Distribution, diurnal vertical, of Gonyostomum semen in a bog pond, 286.
Dogfish ear, sensory hairs, 429.
Dogfish, reactivation by cutting of severed melanophore nerves, 255.
DUBUY, H. G. Separation of the conducting and contractile elements in the retractor muscle of Thyone briareus (abstract), 408.

EAR, dogfish, sensory hairs of, 429.
Egg, membranes and germinal vesicle, Sabellaria vulgaris, 46.
Elodea cells, free calcium in action of stimulating agents on, 306.
Environment and sex in oviparous oyster, Ostrea virginica, 353.
Environment, conditions of, in a bog pond, and diurnal vertical distribution of Gonyostomum semen, 286.
ERIKSSON-QUENSEL, INGA-BRITTA, AND THE SVEDBERG. The molecular weights and pH-stability regions of the hemocyanins, 498.
Excretion of inorganic phosphate in sculpin, 360.
——, rôle of blood cells, in ascidians, 249.

FIGGE, FRANK H. J. The effect of some oxidation-reduction indicator dyes (phenol indophenol) on the eyes and pigmentation of normal and hypophysectomized amphibians (abstract), 393.
FISH, CHARLES J. The biology of Oithona similis in the Gulf of Maine and Bay of Fundy, 168.
FISHER, K. C., AND J. A. CAMERON. The effect of light on the CO-poisoned embryonic Fundulus heart (abstract), 404.
Fission rate, effects of heavy water on, of Uroleptus mobilis, 199.
FOX, DENIS L. *See* Young and Fox, 217.
FRENCH, C. S. Efficiency of photosynthesis in purple bacteria (abstract), 394.

GASTRULATION, effect of alcohol on, in Arbacia, 413.
GEORGE, W. C. The rôle of blood cells in excretion in ascidians, 249.
Germinal vesicle, of egg of Sabellaria vulgaris, 46.
GIER, HERSCHEL T. The morphology and behavior of the intracellular bacteroids of roaches, 433.
GLASSMAN, H. N. *See* Jacobs, Glassman and Parpart (abstract), 401.
Gonyostomum semen, diurnal vertical distribution of, and environmental conditions, 286.
GRAFFLIN, ALLAN L. Renal function in marine teleosts. IV. The excretion of inorganic phosphate in the sculpin, 360.
GRAVE, B. H., AND J. SMITH. Hermaphroditism in Mollusca (abstract), 407.
Gut, structure and function, in surf perches with reference to carotenoid metabolism, 217.
GUYER, M. F., AND PEARL E. CLAUS. Recovery changes in transplanted anterior pituitary cells stratified in the ultra-centrifuge, 462.

HAIRS, sensory, of dogfish ear, 429.
HARTLINE, H. K. *See* Lucké, Ricca and Hartline (abstract), 397.

——, —. —. The discharge of impulses in the optic nerve fibers of the eye of Pecten irradians (abstract), 403.
HARVEY, ETHEL BROWNE. Parthenogenetic merogony or cleavage without nuclei in Arbacia punctulata, 101.
HARVEY, E. N. *See* Hunter and Harvey (abstract), 396.
Heavy water, effects on fission rate of Uroleptus mobilis, 199.
HEILBRUNN, L. V. Protein lipid binding in protoplasm, 299.
Hemocyanins, molecular weights and pH-stability regions of, 498.
HENSHAW, P. S. The question of recovery from X-ray effects in Arbacia sperm (abstract), 399.
HÖRSTADIUS, S. Investigations on determination in the early development of Cerebratulus (abstract), 406.
HUNNINEN, A. V., AND R. WICHTERMAN. Hyperparasitism: A species of Hexamita (Protozoa, Mastigophora) found in the reproductive systems of Deropristis inflata (Trematoda) of marine eels (abstract), 409.
HUNTER, F. R., AND E. N. HARVEY. The effect of lack of oxygen on the permeability of the egg of Arbacia punctulata (abstract), 396.
HUNTER, LAURA N. Some nuclear phenomena in the Trichodina (Protozoa, Ciliata, Peritrichida) from Thyone briareus (Holothuroidea) (abstract), 406.

IRVING, LAURENCE. Physiological adjustments to diving in the beaver (abstract), 391.

JACOBS, M. H. *See* Parpart and Jacobs (abstract), 400.
——, —. —., H. N. GLASSMAN AND A. K. PARPART. Further studies on specific physiological properties of erythrocytes (abstract), 401.

KEIL, ELSA M., AND F. J. M. SICHEL. The injection of aqueous solutions, including acetylcholine, into the isolated muscle fiber (abstract), 402.
KELLEY, ROBERT W. *See* Saxton, Schmeckebier and Kelley, 453.
KELTCH, ANNA K., G. H. A. CLOWES AND M. E. KRAHL. The respiratory effects exerted by certain organic compounds in relation to their molecular structure (abstract), 399.
KINDRED, J. E. An interpretation of the secondary lymphoid nodules in the albino rat (abstract), 390.
KING, R. L. *See* Beams and King, 188.
KOONZ, CARL H. Some unusual cytological phenomena in the spermatogenesis of a haploid parthenogenetic hymenopteran, Aenoplex smithii (Packard), 375.
KOPAC, M. J. Interfacial films between oil and cytoplasm (abstract), 398.
KRAHL, M. E. *See* Keltch, Clowes and Krahl (abstract), 399.
——, —. —., G. H. A. CLOWES AND J. F. TAYLOR. Action of metabolic stimulants and depressants on cell division at varying carbon dioxide tensions (abstract), 400.

LACKEY, J. B. Some freshwater Protozoa with blue chromatophores, 492.
LIPMAN, HARRY J. The phosphatase content of the developing chick embryo (abstract), 386.
LUCAS, ALFRED M. Nerve cells without central processes in the fourth spinal ganglion of the frog (abstract), 387.
LUCKÉ, B., R. RICCA AND H. K. HARTLINE. Comparative permeability to water and certain solutes of the egg cells of three marine invertebrates (Arbacia, Cumingia and Chaetopterus) (abstract), 397.

MACDOUGALL, MARY S. Cytological studies of the genus Chilodonella, III. The conjugation of Chilodonella labiata, variation? (abstract), 410.
Marine Biological Laboratory, thirty-eighth report, 1.
MARTIN, EARL A. Asexual reproduction in Dodecaceria fimbriatus (abstract), 396.
MAZIA, DANIEL, AND JEAN M. CLARK. Free calcium in the action of stimulating agents on Elodea cells, 306.
Melanophore nerves, reactivation by cutting, in dogfish, 255.
—— system, physiology of, in catfish, 259.

Melanosis, cutaneous, in lungfishes, 282.
Membranes and germinal vesicle of egg of Sabellaria vulgaris, 46.
Merogony, parthenogenetic, or cleavage without nuclei in Arbacia punctulata, 101.
METZ, C. W. Effects of mechanical distortion on the structure of salivary gland chromosomes, 238.
MILLS, K. O. *See* Cameron and Mills (abstract), 405.
Mitotic spindle, components of, 469.
—— ——, effect of ultracentrifuging on, in chick embryonic cells, 188.
Mnemiopsis leidyi, regeneration in, 421.
Molecular weights and pH-stability regions of the hemocyanins, 498.
Mosquito larvae, utilization of solutes by, 343.
Mustelus, reactivation by cutting of severed melanophore nerves, 255.

NITRITE in the sea, occurrence and significance of, 133.

OITHONA similis, biology of, in Gulf of Maine and Bay of Fundy, 168.
Oyster, environment and sex in, 353.
Oxygen tension, influence on multiplication of bacteria, 324.

PARKER, G. H. The reactivation by cutting of severed melanophore nerves in the dogfish, Mustelus, 255.
PARPART, A. K. *See* Jacobs, Glassman and Parpart (abstract), 401.
——, —. —. The permeability of the erythrocytes of the ground-hog (abstract), 387.
——, —. —., AND M. H. JACOBS. Paradoxical osmotic volume changes in erythrocytes (abstract), 400.
Parthenogenetic merogony, or cleavage without nuclei, in Arbacia punctulata, 101.
Phosphate, inorganic, excretion of, in sculpin, 360.
pH-stability regions of the hemocyanins, 498.
Pituitary, anterior, cells, recovery changes in, after stratification in ultra-centrifuge, 462.
Program and abstracts of scientific papers presented at the Marine Biological Laboratory in the summer of 1936, 386.
Protein lipid binding in protoplasm, 299.

RAKESTRAW, NORRIS W. The occurrence and significance of nitrite in the sea, 133.
Reactivation by cutting of severed melanophore nerves in dogfish, 255.
Recovery changes in transplanted anterior pituitary cells stratified in the ultra-centrifuge, 462.
Regeneration in Mnemiopsis leidyi Agassiz, 421.
Renal function in marine teleosts: excretion of inorganic phosphate in sculpin, 360.
RICCA, R. *See* Lucké, Ricca and Hartline (abstract), 397.
RIETHMILLER, GRACE. *See* Wolf and Riethmiller (abstract), 391.
Roaches, intracellular bacteroids of, 433.
RUGH, ROBERTS. A quantitative analysis of the anterior pituitary-ovulation relation in the frog (abstract), 389.

SABELLARIA vulgaris, membranes and germinal vesicle of egg of, 46.
Salivary gland chromosomes, effects of mechanical distortion on structure of, 238.
SANDOW, ALEXANDER. Diffraction patterns of striated muscle and sarcomere behavior during contraction (abstract), 392.
SASLOW, G. Prevention of edema in frog perfusions in the absence of serum proteins (abstract), 404.
SAXTON, JOHN A., MARY M. SCHMECKEBIER AND ROBERT W. KELLEY. Autogenous transplantations of pigmented and unpigmented ear skin in guinea pigs, 453.
SCHECHTER, VICTOR. Comparative hypotonic cytolysis of several types of invertebrate egg cells and the influence of age (abstract), 410.
SCHMECKEBIER, MARY M. *See* Saxton, Schmeckebier and Kelley, 453.
SCHMITT, F. O., R. S. BEAR AND J. Z. YOUNG. Some physical and chemical properties of the axis cylinder of the giant axons of the squid, Loligo pealii (abstract), 402.
SCHOEPFLE, G., AND J. Z. YOUNG. The structure of the eye of Pecten (abstract), 403.
Sculpin, excretion of inorganic phosphate, 360.

Sex and environment in the oviparous oyster, Ostrea virginica, 353.
Sexual phases, functional, sequence of, in Teredo, 122.
SICHEL, F. J. M. *See* Keil and Sichel (abstract), 402.
——, —. —. —., AND A. C. BURTON. A kinetic method of studying surface forces in the egg of Arbacia (abstract), 397.
SMITH, G. M., AND C. W. COATES. Cutaneous melanosis in lungfishes (Lepidosirenidae), 282.
SMITH, J. *See* Grave and Smith (abstract), 407.
Solutes, utilization by mosquito larvae, 343.
SPEIDEL, C. C. Experiments on the contractile substance of muscle fibers (abstract), 401.
Spermatogenesis, cytological phenomena of a haploid parthenogenetic hymenopteran, Aenoplex smithii (Packard), 375.
STEINBACH, H. BURR. Effects of salts on the injury potentials of frog's muscle (abstract), 388.
Stimulating agents, free calcium in action of, on Elodea cells, 306.
STUNKARD, H. W. Notes on life cycles of digenetic trematodes (abstract), 411.
SUGIYAMA, M. *See* Dan, Yanagita and Sugiyama (abstract), 395.
SVEDBERG, THE. *See* Eriksson-Quensel and Svedberg, 498.

TAYLOR, J. F. *See* Krahl, Clowes and Taylor (abstract), 400.
Teredo, sequence of functional sexual phases, 122.
TRAGER, WILLIAM. The utilization of solutes by mosquito larvae, 343.
Transplantations, autogenous, of pigmented and unpigmented ear skin in guinea pigs, 453.
TYLER, ALBERT. On the energetics of differentiation, III, IV, 59, 82.

ULTRA-centrifuge, recovery changes in transplanted anterior pituitary cells stratified in, 462.
——, effect on chick embryonic cells, with reference to "resting" nucleus and mitotic spindle, 188.
Uroleptus mobilis, effect of heavy water on fission rate and life cycle, 199.

WATERMAN, A. J. Inhibition of gastrulation in Arbacia with nickel chloride (abstract), 411.
——, —. —. The effect of alcohol on gastrulation in Arbacia, 413.
——, —. —. The membranes and germinal vesicle of the egg of Sabellaria vulgaris, 46.
WICHTERMAN, R. Division and conjugation in Nyctotherus cordiformis (Ehr.) Stein (Protozoa, Ciliata) with special reference to the nuclear phenomena (abstract), 412.
——, —. *See* Hunninen and Wichterman (abstract), 409.
WOLF, E. ALFRED, AND GRACE RIETHMILLER. Studies in calcification: III. The shell of the hen's egg (abstract), 391.

YANAGITA, T. *See* Dan, Yanagita and Sugiyama (abstract), 395.
YOUNG, J. Z. *See* Schmitt, Bear and Young (abstract), 402.
——, —. —. *See* Schoepfle and Young (abstract), 403.
——, ROBERT T., AND DENIS L. FOX. The structure and function of the gut in surf perches (Embiotocidae) with reference to their carotenoid metabolism, 217.

ZOBELL, CLAUDE E. Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces, 324.

MARINE BIOLOGICAL LABORATORY SUPPLY DEPARTMENT

LIVING MARINE MATERIAL

For a number of years the Supply Department has been furnishing Living Marine Materials. From experience it has been found that during the period between the first of November and the end of February these live animals and plants can be shipped with the most success. This service has proven to be of great value to both instructor and student, particularly to those who are located at some distance from the sea coast.

MARINE AQUARIA SETS

The following marine sets will be supplied during November, December, January, and February. **We assume responsibility for shipments going as far west as the Mississippi and as far south as Georgia, and guarantee to deliver the animals in good condition.** Although we have been successful in shipping live Marine Aquaria Sets to New Mexico, Texas, Missouri, North Dakota, and Iowa, we do not guarantee safe delivery except as stated above.

Set I. Live Marine Material for five-gallon Aquarium. Includes the following:

5 gallons Sea water
2 small Metridium (Sargartia may be substituted)
2 small Starfish
1 Hermit Crab
2 small Sea Urchins
1 small Thyone
2 small Mytilus
2 Littorina
Green algae (Ulva when available)

Price per set **$5.00**

Set II. Live Marine Material for ten-gallon Aquarium. Includes the following:

10 gallons Sea water
4 small Metridium
2 small Starfish
1 Red Starfish (Henricia)
Green algae (Ulva when available)
3 small Sea Urchins
2 small Thyone
2 small Mytilus
Balanus
2 Hermit Crabs
Littorina or Urosalpinx
1 Nereis
1 small Limulus
Astrangia
1 Brittle Starfish

Price per set **$10.00**

Other marine animals not listed can be supplied on short notice. Substitutions can be made on any of the above sets. Prices on larger sets will be gladly given upon request.

All shipping containers furnished at cost. Credit of $1.50 will be given for each carboy returned by prepaid express.

The sea water for these sets is shipped a day or so in advance of the materials, together with instructions for the care of the living forms, in order that the aquarium may be set up and ready for the specimens when they arrive.

If there is any further information desired regarding the above sets, we are very glad to furnish same.

MARINE BIOLOGICAL LABORATORY

WOODS HOLE, MASS.

CONTENTS

Page

WATERMAN, A. J.

The Effect of Alcohol on Gastrulation in Arbacia 413

COONFIELD, B. R.

Regeneration in Mnemiopsis leidyi, Agassiz 421

BOWEN, R. E.

Sensory Hairs of the Dogfish Ear 429

GIER, HERSCHEL T.

The Morphology and Behavior of the Intracellular Bacterioids of Roaches 433

SAXTON, JOHN A., JR., MARY M. SCHMECKEBIER AND ROBERT W. KELLEY

Autogenous Transplantations of Pigmented and Unpigmented Ear Skin in Guinea Pigs 453

GUYER, M. F., AND PEARL E. CLAUS

Recovery Changes in Transplanted Anterior Pituitary Cells Stratified in the Ultra-Centrifuge 462

CAROTHERS, E. ELEANOR

Components of the Mitotic Spindle with Especial Reference to the Chromosomal and Interzonal Fibers in the Acrididæ 469

LACKEY, JAMES B.

Some Fresh Water Protozoa with Blue Chromatophores 492

ERIKSSON-QUENSEL, INGA-BRITTA, AND THE SVEDBERG

The Molecular Weights and pH-Stability Regions of the Hemocyanins 498

INDEX 548